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Award Number: W81XWH-12-1-0504

TITLE: Novel Target for Ameliorating Pain and Other Problems after SCI: Spontaneous Activity in Nociceptors

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REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	15September2012–14September2013

4. TITLE AND SUBTITLE
Novel Target for Ameliorating Pain and Other Problems after SCI: Spontaneous Activity in Nociceptors

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0504

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas Health Science Center

Houston TX 77030-5400

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of the project is test the hypothesis that interventions that reduce the function of a Na^+ ion channel, Nav1.8, that is selectively expressed in primary afferent neurons (especially nociceptors) ameliorate chronic pain and reflex hypersensitivity caused by traumatic spinal cord injury (SCI). The first phase of the project has largely been accomplished, despite several problems that had to be solved. Major results were a confirmation that Nav1.8 protein expression is selectively knocked down by intrathecal injection of antisense oligodeoxynucleotides targeting Nav1.8 mRNA, and demonstrations that this knockdown results not only in a reversal of mechanical and heat hypersensitivity of hindlimb withdrawal reflexes after SCI, but also in significant amelioration of ongoing, spontaneous pain. The existence of spontaneous pain in rodents after traumatic SCI and its dependence on Nav1.8 function were shown for the first time, using an operant conditioned place preference paradigm and an analgesic, retigabine, known to potently suppress electrical activity in primary nociceptors. These results suggest a promising new approach to treat SCI pain.

15. SUBJECT TERMS
Spinal cord injury, neuropathic pain, primary nociceptors, Nav1.8 sodium channels, conditioned place preference

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	65	USAMRMC

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Introduction

The purpose of this project is to test a novel approach to treating chronic pain and other complications of spinal cord injury (SCI) using a preclinical rat model. Over 40,000 veterans have SCI, as well as many active members of the armed services, and a majority of these people endure intractable pain and potentially related chronic problems such as gastrointestinal dysfunction for the rest of their lives. Most investigators have assumed that the critical mechanisms driving SCI pain are located within the central nervous system (CNS) and involve direct effects of the injury and/or associated neuroinflammation on pain pathways (Finnerup and Baastrup, 2012; Walters, 2012; see also attached manuscript, Walters, *Exp Neurol*, submitted). Early evidence that primary sensory neurons, and especially primary nociceptors, are involved in neuropathic SCI pain came from observations of enhanced nociceptor growth after SCI (Ondarza et al., 2003; Bedi et al., 2012). Primary nociceptors are the first neurons within pain pathways and thus their electrical activity leads to the conscious sensation of pain as well associated reflex responses. These sensory neurons are specialized for the detection of bodily injury and inflammation and are normally electrically silent, firing action potentials only when their peripheral branches are activated by stimuli that can produce pain (fortunately, an infrequent occurrence for most people).

The present grant enables rigorous tests of our hypothesis that prominent aspects of chronic pain and hypersensitivity caused by SCI can be ameliorated effectively by interventions that selectively block spontaneous electrical activity in primary nociceptors. Three years ago we reported (Bedi et al., 2010) the unexpected discovery that primary nociceptors in rats that have received a contusive spinal injury (controlled experimental bruising of the spinal cord) months earlier continuously fire action potentials without any extrinsic stimulation ("spontaneous activity," SA), even when the recorded nociceptor is removed from the body and isolated from all other cells. Electrical activity in any nociceptor would be expected to excite pain pathways and thereby produce or promote pain sensations, and so it was not surprising to find that this chronic nociceptor SA was closely correlated with behavioral measures of pain; animals exhibiting pain showed a high incidence of nociceptor SA whereas apparently pain-free animals did not. More direct evidence that activity in primary nociceptors helps to maintain SCI pain came from our finding that antisense knockdown of TRPV1 channels or pharmacological blockade of TRPV1 channels -- which are expressed most abundantly in nociceptors (Caterina et al., 2000; Lauria et al., 2006) -- reduced SA after SCI and caused a dramatic reversal of reflex hypersensitivity (Wu et al., 2013, see Appendix). Importantly, the nociceptors exhibiting SA after SCI possess an ion channel, Nav1.8, that in the nervous system is only expressed by primary sensory neurons, and primarily in nociceptors (Liu and Wood, 2011; Shields et al., 2012). We found that a drug that selectively blocks Nav1.8 channels also blocks SA in nociceptors after SCI.

These recent discoveries led directly to the hypothesis and associated experiments in this project. We are testing the prediction that interventions that reduce Nav1.8 function -- specifically antisense knockdown of Nav1.8 expression and inhibition of Nav1.8 channels using two different drugs -- will reduce chronic pain and visceral hypersensitivity (as well as SA and hyperexcitability in nociceptors) after SCI. To model chronic dysfunction after SCI, animals are tested 6 to 12 weeks after injury. A novel and potentially important part of our experimental design is to develop and apply an operant measure of ongoing, spontaneous pain after SCI that will provide a much more relevant model of neuropathic SCI pain than has been available in the hyperreflexia measures that almost all previous studies of SCI pain have depended on.

Body

This project had four aims that were addressed experimentally via two major tasks. Task 1 addressed the first three aims, which were i) (Tasks 1b,c) to test the hypothesis that chronic behavioral hypersensitivity in a rat contusive SCI model is reduced by blocking spontaneous activity (SA) in nociceptors via specific reduction in Nav1.8 activity, ii) (Tasks 1d,e) to test whether this behavioral hypersensitivity can be reduced briefly or for a prolonged period by single or repeated application of a less specific but more affordable and clinically promising drug that reduces Nav1.8 activity, and iii) (Tasks 1b-e) to test the prediction that chronic visceral hypersensitivity after SCI can be reduced by reducing the activity and/or expression of Nav1.8 channels. Task 2 addressed the fourth and most important aim, which was to test the hypothesis that nociceptor SA (dependent on Nav1.8 channel function) drives chronic spontaneous pain after SCI. No animal models have adequately examined spontaneous SCI pain, which arguably is the worst type of neuropathic SCI pain. Moreover, no investigations of any form of spontaneous pain have attempted to assess contributions of chronic activity in nociceptors to the pain. During Year 1 we have made major progress towards these goals, completing the most technically demanding part of the project (antisense oligodeoxynucleotide injections through an intrathecal catheter in Task 1b) and finding an exciting answer to the most important question in the project (showing that Nav1.8 knockdown decreases signs of spontaneous pain in Task 2a). These findings are described below, and are being combined into a single manuscript that will soon be submitted to the *Journal of Neuroscience*. We have also encountered obstacles that have slowed some of our efforts, and these are also described below.

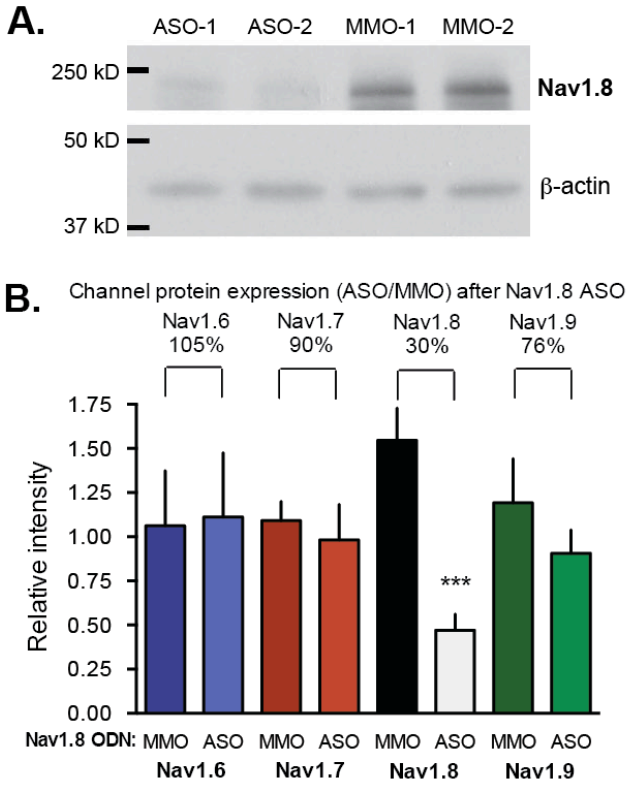

Fig. 1. Intrathecal delivery of antisense ODN (ASO) to Nav1.8 mRNA selectively reduces expression of Nav1.8 protein in L4/L5 DRGs 4-6 weeks after SCI at T10. A. Examples of Western blots from 4 animals injected with Nav1.8 ASO or MMO (mismatch) ODNs. B. Mean blot intensities relative to β-actin for different Na^+ channel proteins from SCI animals given Nav1.8 ASO or MMO injection. The animal n's for each group were 5 Nav1.6, 5 Nav1.7, 6 Nav1.8, and 6 Nav1.9. Only Nav1.8 showed a significant decrease in expression, indicating considerable specificity in Nav1.8 ASO effects. From Yang et al., in preparation.

Definitions of Major Tasks

Task 1 is to test the effects of four different Nav1.8 interventions on **hypersensitivity to bodily stimulation** (somatic and visceral reflex sensitivity and evoked pain). These Nav1.8 interventions are a) antisense knockdown, b) brief block with the drug, A-803467, c) brief block with the drug, ambroxol, and d) prolonged block with ambroxol. **Task 2** is to test the effects of the same Nav1.8 interventions on **spontaneous pain** in these animals (i.e., Tasks 1 and 2 are designed to extract different information from the same animals). After completing Task 1a (obtaining institutional and DoD approvals for our animal use protocols), all of the experimental manipulations have been applied to both Tasks, which in Year 1 have focused entirely on Tasks 1b and 2a.

Task 1b - Investigate effects of knocking down Nav1.8 expression on SCI-induced behavioral hypersensitivity and nociceptor activity

Task 1b Accomplishments

Task 1b investigates effects of knocking down the expression of Nav1.8 protein by intrathecal antisense oligodeoxynucleotide (ODN) treatment on behavioral hypersensitivity, while Task 2a investigates effects in the same animals on an operant measure of spontaneous pain. Task 1b had two purposes. One was to extend our pilot studies implicating a major role for Nav1.8 channels in maintaining behavioral hypersensitivity chronically after SCI. The second was to optimize our methods so that the important preclinical studies using drugs in Tasks 1c-1e and 2b-2d can be performed as efficiently as possible.

Our first step in Task 1b was to validate our knockdown procedure by using Western blot analysis to determine whether repeated intrathecal injection of an ODN antisense (ASO) to Nav1.8 mRNA selectively reduces the expression of Nav1.8 protein in lumbar dorsal root ganglia (DRGs) 4 to 6 weeks after spinal contusion at segment T10. As shown in Fig. 1, ASO treatment caused a significant, 70% reduction in Nav1.8 protein compared to the levels determined after treatment with mismatched ODNs (MMO). This result was similar to that previously described using intrathecal injection of the same ASO and MMO sequences after peripheral nerve injury (Lai et al., 2002). However, no studies had shown that this ASO sequence selectively knocked down Nav1.8 and not other voltage-gated Na^+ channels. Importantly, we found that three other Na^+ channels, Nav1.6, Nav1.7, and Nav1.9, failed to show significant reduction in protein expression after intrathecal injection of Nav1.8 ASO. We also compared Nav1.8 protein expression in lumbar DRGs from SCI, sham-treated, and naïve animals and found that, in the absence of ODN injection, SCI caused a significant increase in Nav1.8 expression (Fig. 2). This is the first demonstration of Nav1.8 upregulation after any form of central neural injury.

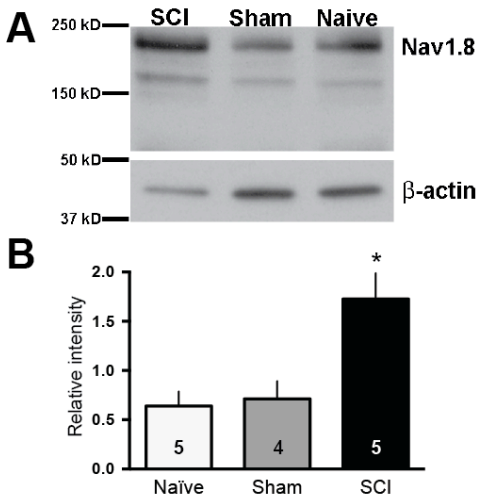

Fig. 2. Nav1.8 in L4/L5 DRGs is upregulated 4-6 weeks after SCI at T10. Western blot analysis revealed a significant elevation of blot intensity (relative to beta actin) in DRGs taken from SCI animals compared to either sham-treated animals or naive animals. From Yang et al., in preparation.

We now have now completed our study of the effects of Nav1.8 knockdown on mechanical and heat hypersensitivity 6-12 weeks after SCI. The effects on mechanical hypersensitivity of the hindlimbs are shown in Fig.3. The mean (± SEM) measures of sensitivity to mechanical testing before and after SCI reveal, as we predicted (Bedi et al., 2010; Wu et al., 2013), that mechanical sensitivity increased after SCI, as indicated by a decrease in the von Frey filament force needed to evoke hindlimb withdrawal post-SCI. Importantly, the animals treated with Nav1.8 ASO showed a reversal of mechanical hypersensitivity after treatment, with the thresholds being significantly higher than those in the animals treated with MMO.

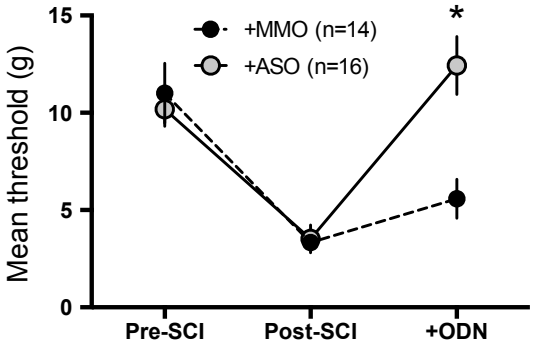

Fig. 3. Mechanical hypersensitivity 6-10 weeks after SCI is reversed by Nav1.8 knockdown. The threshold for eliciting a hindlimb withdrawal response with von Frey filament stimulation was significantly higher following Nav1.8 ASO treatment than MMO treatment. From Yang et al., in preparation.

The effects of SCI on heat sensitivity have been more complicated. When all animals tested so far are included in the analysis, SCI-induced heat hypersensitivity occurs (Fig.4A and B) as we previously reported (Bedi et al., 2010; Wu et al., 2013 – see appendix).

However, in contrast to our early pilot data (Fig. 2 in the grant proposal), subsequent reversal of SCI-induced heat hypersensitivity by Nav1.8 ASO was not statistically significant (Fig.4A). We

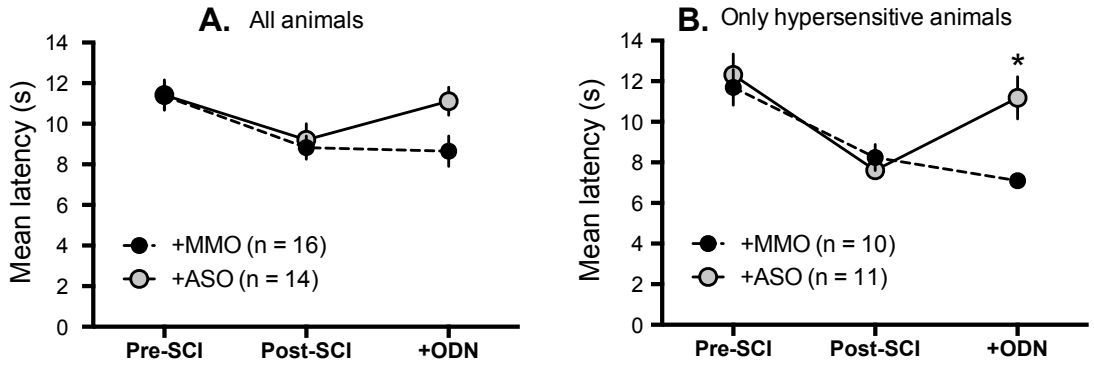

Fig. 4. Heat hypersensitivity 6-10 weeks after SCI is reversed by Nav1.8 knockdown. A. When all animals were included in the analysis, the latency for eliciting a hindlimb withdrawal response to a radiant heat stimulus directed to the plantar surface of each hindpaw (averaged) was significantly lower after SCI, but was not significantly higher following Nav1.8 ASO treatment compared to MMO treatment. B. After excluding animals that failed to exhibit heat hypersensitivity after SCI, the ASO effect was statistically significant. Yang et al., in preparation.

noticed that fewer animals developed heat hypersensitivity than mechanical hypersensitivity under the conditions we have been using in the current studies. When the analysis includes only the animals that developed heat hypersensitivity after SCI, Nav1.8 ASO reversed heat hypersensitivity, as judged by the significant difference between the latencies of animals after ASO and after MMO treatment (Fig. 4B).

We have verified that Nav1.8 ASO treatment reduces spontaneous activity in subsequently dissociated small DRG neurons, and reduces TTX-resistant currents (largely mediated by Nav1.8 channels) 6 to 14 weeks after SCI (Fig.5). This strongly suggests that a reduction of nociceptor activity, including SA, by Nav1.8 knockdown is at least partly responsible for the suppressive effects found on SCI-induced behavioral hypersensitivity.

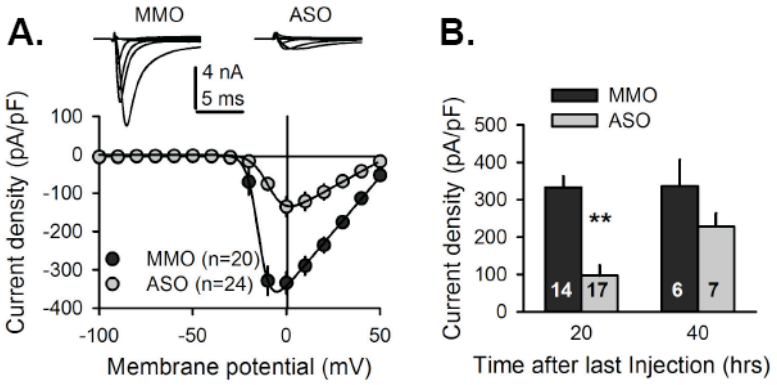

Fig. 5. Suppression of TTX-resistant current in dissociated nociceptors by prior knockdown of Nav1.8 channels *in vivo*. A. I-V relationships under voltage clamp were determined with whole cell patch clamp 20 h after the last intrathecal ODN injection at the lumbar enlargement. B. Comparison of currents at 0 mV 20 and 40 h after ODN injection. From Yang et al., in preparation.

Task 1b Problems

In addition to examining the effects of Nav1.8 knockdown on SCI-induced hyperreflexia, we had proposed to examine the effects on two operant measures that would capture the aversive and cognitive aspects of evoked pain: the place/escape avoidance paradigm (PEAP) and the mechanical conflict-avoidance paradigm (MCAP). Because of the large number of behavioral tests given to each animal in this study, and because early results suggested that the PEAP and CPP (see Task 2b) might be sufficiently similar to each other for the PEAP to interfere with CPP performance, we decided to drop the PEAP test. In addition, we found that the supraspinal test site (at the base of the skull) was close enough to the incision site for the intrathecal catheter to be affected by local sensitization remaining after the incision. Furthermore, the PEAP test probably provides information about evoked pain sensitivity that is redundant with that provided by the MCAP operant test. We had not used the MCAP test before, and there was only one

published paper describing its use. Therefore, we began with a complex MCAP protocol involving the successive use of increasingly higher probes to find the optimal probe height to provide painful stimulation that would adequately oppose the aversiveness of the bright light in the start chamber. Some of the results obtained thus far are promising but are not ready for inclusion in a formal study because of ongoing improvements we are making to the protocol. We are engaged in discussions with the designers of the MCAP test system (Coy Lab Products) and with our colleague, Dr. Susan Carlton at the University of Texas Medical Branch in Galveston, who is also working to optimize the MCAP test. We will have an improved procedure ready for formal use in the the drug studies (Tasks 1c-1e and 2b-2d) that are about to begin.

Aim 3 of the proposal was to test the prediction that chronic visceral hypersensitivity after SCI can be reduced by reducing the activity and/or expression of Nav1.8 channels. Two problems forced us to drop this aim from Task 1b. One problem was the unexpected departure of a member of Dr. Hongzhen Hu's laboratory who was assisting with the collection and analysis of the visceromotor data. In addition, Dr. Hu had to take prolonged time away from the visceromotor tests because of illness in his family. This slowed the collection and then the analysis of the visceromotor data. However, both another colleague of Dr. Hu, Dr. Jialie Luo, and Dr. Qing Yang in the Walters lab are now performing and analyzing pilot experiments, and these will be conducted formally in Tasks 1c-e.

The larger problem for assessing visceral hypersensitivity (and other forms of hypersensitivity) was the cumulative stress on the rats from multiple surgeries. In Task 1b the visceromotor testing required a third surgery on animals that had already been stressed by two prior surgeries (one for SCI or sham treatment, and then one to implant the intrathecal catheter). Given the complications encountered with just two surgeries, we decided that visceromotor testing after both SCI and subsequent intrathecal catheterization was not sufficiently informative to justify the additional stress and suffering in the animals. Visceromotor testing will be done in Tasks 1c-e, which do not involve intrathecal catheterization. The most challenging unanticipated problem was the additional attrition of rats and behavioral complications associated with later insertion of the intrathecal catheter. In our preliminary studies the catheter was inserted approximately 4 weeks after SCI and ~80% of the animals survived without obvious neurological symptoms caused by the catheterization, and the heat and mechanical hypersensitivity induced by SCI appeared indistinguishable from that produced by SCI in the absence of catheterization. The standard method we use (see Yaksh and Rudy, 1976) involves insertion at the atlanto-occipital joint and threading of the catheter along the spinal canal, through the T10 injury site, to the lumbar enlargement. Given the much higher attrition rate and weaker expression of reflex hypersensitivity after SCI in the SCI animals after later catheter insertion, we concluded that an interaction of prior SCI with the catheterization procedure compromises the health and reflex behavior of the rats. Because spinal contusion induces syrinx formation and scarring that continue to develop long after spinal trauma, we suspect that catheter insertion at later times is more likely to cause additional injury to the spinal cord and surrounding tissue, causing greater mortality and problems affecting the sensitivity of the reflexes we test. This will not be a problem for Tasks 1c-1e and 2b-2d, which involve systemic drug application without any catheterization (similar systemic drug application with the TRPV1 blocker, AMG9810, worked well in our study by Wu et al. -- see reference at end of report). For much of Task 1 we have reduced the time before inserting the catheter from 11 weeks post-SCI to 6 weeks post-SCI. This means that behavioral and other tests are conducted 7 to 10 weeks post-SCI, which is still a period considered chronic for rodent SCI models. This change has reduced the attrition rate and has increased the incidence of reflex hypersensitivity after SCI.

Task 2a - Investigate effects of Nav1.8 knockdown on spontaneous pain after SCI

Task 2a Accomplishments

Because spontaneous pain after SCI is the major pain-related complaint of SCI patients, a demonstration of spontaneous pain in this rat model of SCI and its amelioration by interference with Nav1.8 function would be the most important achievement of this project. The conditioned place preference (CPP) test is now the standard measure of ongoing or spontaneous pain in

animal models, but its use to investigate neuropathic SCI pain has been restricted to a single report (Davoody et al., *J Pain,* 12:868, 2011) using a model (a focal electrolytic lesion in the cervical spinal cord) that has more limited clinical significance than models of traumatic SCI, such as spinal contusion. The CPP test assumes that an animal in pain will notice and remember a chamber in which it experiences less pain (because of prior injection of an effective analgesic). Thus, we asked whether animals in pain after T10 contusion would spend more time in a chamber in which they had spent three daily 1-hour periods experiencing analgesic effects of retigabine than a chamber paired with vehicle injection. Retigabine, a KCNQ potassium channel blocker, had been shown by others to have analgesic properties and we found that retigabine strongly suppressed SA in nociceptors *in vitro* and *in vivo*. To show that the SCI-induced spontaneous state was effectively aversive, we paired the analgesic injection with placement in an innately aversive white chamber and paired vehicle injection with the innately preferred black chamber. Each animal was tested by being placed in the intermediate grey chamber, where it could choose to stay in place or enter the adjoining black or white chamber. We had anticipated delays with this part of the project because we had no preliminary data (until this grant was funded we lacked funds to purchase an automated CPP box). Moreover, no CPP models anywhere had used retigabine as an analgesic for CPP. Thus, we spent some time optimizing conditions (especially the timing of retigabine application relative to placing the animal in the chamber to be conditioned). Figure 6A shows that, even after partial optimization of the CPP test for SCI pain, we found a significant effect of SCI on this measure of spontaneous pain. After being initially placed in the middle grey chamber, naive and sham-treated animals spent less time in the white, retigabine-paired chamber than in the black, vehicle-paired chamber. In contrast, SCI animals spent significantly more time in the white, retigabine-paired chamber. Importantly, MMO-treated SCI animals also preferred the white chamber, whereas the ASO-treated SCI animals showed no preference for the white, retigabine-paired chamber (Fig. 6B), suggesting that they were not experiencing spontaneous pain after injection of retigabine during the conditioning phase. Supporting the conclusion that SCI produces chronic spontaneous pain in rats, preliminary results indicated that CPP can also be produced by pairing the white chamber with another potential analgesic, a specific TRPV1 channel inhibitor, AMG9810, (n = 5, data not shown). These early results, in combination with the evidence we obtained for the importance of TRPV1 channels in driving neuropathic SCI pain (Wu et al., 2013), indicate that AMG9810 is as good as and perhaps even better than the KQNQ channel blocker, retigabine, for conditioning place preference. The finding that a second drug that inhibits spontaneous activity in primary

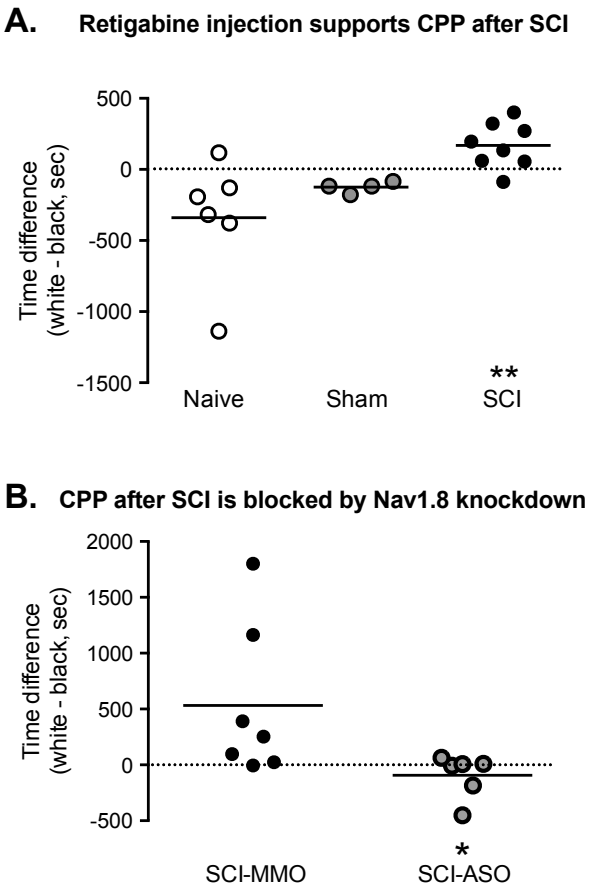

Fig. 6. Evidence that SCI induces chronic spontaneous pain that can be ameliorated by Nav1.8 knockdown. A. SCI animals spent more time in the white, retigabine-paired chamber than did sham-treated or naive animals after CPP training. In this first study the conditioning interval between intraperitoneal retigabine injection and placement in the white chamber was 30 min, and the chambers had grid floors. B. ASO knockdown of Nav1.8 caused a significant reduction in the time spent in the white, retigabine-paired chamber compared to that after MMO injection. In this study the interval between retigabine injection and placement in the white chamber was 10 min and the chambers had solid floors. From Yang et al., in preparation.

nociceptors can support CPP after SCI encourages us to begin the remaining tasks with drugs that target Nav1.8 channels in nociceptors.

Task 2a Problems

No significant problems were experienced with the CPP tests. It is worth emphasizing that our CPP results indicate that this measure of spontaneous pain, unlike our measures of reflex sensitivity, is not affected by problems that can occur when an intrathecal catheter is threaded though the T10 SCI region to the lumbar enlargement. This difference suggests that the CPP measure of spontaneous pain may be both a more clinically relevant endpoint than the hyperreflexia measures and a more practical one to use in studies of the mechanisms that maintain chronic pain after SCI. It is less laborious and more objective (being almost completely automated) and probably much less affected by below-level motor and modulatory complications caused either by the original spinal injury or further injury at- and below-level produced during catheterization. To increase the number of SCI animals that we can test with the CPP procedure during the remainder of the study, we are asking to use some of the unspent funds for a second CPP box. This will help to relieve a general problem that has impacted Task 2a, excessive experimental load for project personnel. In the original SOW the PI underestimated the time required to conduct and analyze all of the experiments. Several alterations have been made to make the work load more reasonable without sacrificing important experiments or data. By shortening the total period of the Nav1.8 knockdown experiments we gain flexibility in scheduling so that members of this small laboratory group are no longer compelled to work in the lab every weekend, and the need for occasional vacation time can be met. The very large number of experimental tests not only makes extreme demands on the experimenters' time, but it also increases chances of interference between behavioral tests. Thus, as mentioned above, we have dropped the PEAP test, which had several shortcomings and provided information redundant with the MCAP test. Reduction in time spent conducting other tests will free up time to perform the CPP tests.

Key Research Accomplishments

1. Demonstrated that intrathecal administration of Nav1.8 antisense oligodeoxynucleotide selectively knocks down Nav1.8 protein expression in lumbar DRGs without significantly reducing the expression of other prominent Na^+ channels in the same ganglia.

2. Discovered that SCI increases the expression of Nav1.8 protein in lumbar DRGs.

3. Confirmed that *in vivo* antisense knockdown of Nav1.8 decreases TTX-resistant inward currents subsequently recorded in dissociated DRG neurons.

4. Demonstrated that antisense knockdown of Nav1.8 reverses SCI-induced reflex hypersensitivity to mechanical test stimuli.

5. Demonstrated that antisense knockdown of Nav1.8 reverses SCI-induced reflex hypersensitivity to heat test stimuli.

6. Discovered that SCI induces a chronic, aversive state with the properties of spontaneous pain, by showing the conditioning of place preference using a KCNQ channel opener, retigabine, as an analgesic to relieve the pain.

7. Discovered that antisense knockdown of Nav1.8 prevents the conditioning of place preference with retigabine, providing strong evidence that ongoing activity in primary sensory neurons plays a major role in driving chronic spontaneous pain after SCI.

Reportable Outcomes

Publications (see Appendix)
Wu, Z, Yang, Q, Crook, RJ, O'Neil, RG, Walters, ET (2013) TRPV1 channels make major contributions to behavioral hypersensitivity and spontaneous activity in nociceptors after spinal cord injury. *Pain,* 154:2130–2141.

[In collecting some of the behavioral, electrophysiological, and Western blot data for this paper, Drs. Wu and Yang optimized methods that they also used in concurrent experiments on Nav1.8 knockdown effects described in this annual report. Thus, although no funds from the present grant were used to purchase animals or supplies for the experiments in the Wu et al. paper, the present grant's partial support of these investigators' salaries during the past year permitted them to conduct experiments that benefited both that paper and this project, while not taking effort away from this project.]

Walters, ET (2014) Neuroinflammatory contributions to pain after SCI: roles for central glial mechanisms and peripheral host defense. *Exp Neurol,* submitted.

[This invited review article (peer reviewed) was written while receiving support from this grant. It provides a novel theoretical perspective on neuroinflammatory aspects of SCI pain based on the findings that Nav1.8 knockdown and TRPV1 knockdown ameliorate neuropathic SCI pain. Briefly, SCI is proposed to release signals in the cord and systemic circulation that trigger a host defense response that includes persistent nociceptor activation to drive chronic pain.]

Invited Presentations (in which project findings were presented)

- Mission Connect/TIRR meeting, Houston, TX, February 2013
- Drexel University, Dept. of Neurobiology & Anatomy, Philadelphia PA, March, 2013
- Kentucky Spinal Cord and Head Injury Research Symposium, Lexington KY May, 2013
- Symposium "Primary Afferent Hyperexcitability Drives Chronic Pain", American Pain Society meeting, New Orleans LA, May 2013
- International Spinal Research Trust Network meeting, London, UK, September, 2013

Personnel Paid by this Grant

Edgar T. Walters, Ph.D., PI
Qing Yang, M.D., co-PI
Zizhen Wu, M.D./Ph.D., graduate research assistant and, briefly, research associate
(Dr. Wu's effort ended September 2, 2013)
Julia Hadden, research assistant
(Ms. Hadden began her formal effort on the project September 15, 2013 but received training by helping on this project prior to this start date)

Conclusion

In the first year of this project we have made substantial progress on some of the most technically challenging and clinically promising studies. Although problems were encountered with intrathecal catheterization and visceromotor testing, we confirmed that Nav1.8 channels are selectively knocked down by antisense ODN injection, and that this knockdown results not only in a reversal of mechanical and heat hypersensitivity after SCI, but also an amelioration of spontaneous pain, as indicated by the first successful use of the operant conditioned place preference paradigm after traumatic SCI in a rodent model system.

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Appendix

References denoted by an asterisk (*) above are included in the appendix (following pages).

PAIN® 154 (2013) 2130–2141

www.elsevier.com/locate/pain

TRPV1 channels make major contributions to behavioral hypersensitivity and spontaneous activity in nociceptors after spinal cord injury

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ARTICLE INFO

Article history:
Received 13 March 2013
Received in revised form 18 June 2013
Accepted 24 June 2013

Keywords:
Allodynia
Capsaicin
Dorsal root ganglion
Hyperalgesia
Pain
Spinal contusion

ABSTRACT

Chronic neuropathic pain is often a severe and inadequately treated consequence of spinal cord injury (SCI). Recent findings suggest that SCI pain is promoted by spontaneous activity (SA) generated chronically in cell bodies of primary nociceptors in dorsal root ganglia (DRG). Many nociceptors express transient receptor potential V1 (TRPV1) channels, and in a preceding study most dissociated DRG neurons exhibiting SA were excited by the TRPV1 activator, capsaicin. The present study investigated roles of TRPV1 channels in behavioral hypersensitivity and nociceptor SA after SCI. Contusive SCI at thoracic segment T10 increased expression of TRPV1 protein in lumbar DRG 1 month after injury and enhanced capsaicin-evoked ion currents and Ca^{2+} responses in dissociated small DRG neurons. A major role for TRPV1 channels in pain-related behavior was indicated by the ability of a specific TRPV1 antagonist, AMG9810, to reverse SCI-induced hypersensitivity of hind limb withdrawal responses to mechanical and thermal stimuli at a dose that did not block detection of noxious heat. Similar reversal of behavioral hypersensitivity was induced by intrathecal oligodeoxynucleotides antisense to TRPV1, which knocked down TRPV1 protein and reduced capsaicin-evoked currents. TRPV1 knockdown also decreased the incidence of SA in dissociated nociceptors after SCI. Prolonged application of very low concentrations of capsaicin produced nondesensitizing firing similar to SA, and this effect was enhanced by prior SCI. These results show that TRPV1 makes important contributions to pain-related hypersensitivity long after SCI, and suggest a role for TRPV1-dependent enhancement of nociceptor SA that offers a promising target for treating chronic pain after SCI.

1. Introduction

Chronic neuropathic pain occurs in approximately half of all patients with spinal cord injury (SCI) and can be severely debilitating [26]. Because treatment of neuropathic pain after SCI is often ineffective, there is a pressing need to further define underlying mechanisms so that better therapies can be developed [27]. Mechanisms maintaining neuropathic SCI pain are generally assumed to reside within the central nervous system [26], and this assumption is supported by the association of numerous central alterations with SCI pain. For example, increased expression of excitability-promoting ion channels occurs within the spinal dorsal horn [33] and thalamus [34], various disinhibitory alterations occur in the spinal cord [49,53], dendritic spine remodeling occurs in the spinal cord [66], and reorganization occurs in the somatosensory cortex [76]. In principle, these alterations might be promoted by enhanced input from primary afferent neurons. SCI triggers an enhanced growth state in numerous nociceptors [7,57,61], as well as enhanced sensitivity and spontaneous activity (SA) in their peripheral branches [12]. We found a high incidence of SA and hyperexcitability in cell bodies of probable nociceptors both in vivo and when dissociated from dorsal root ganglia (DRG) long after SCI [8]. Close correlation of SA in small DRG neurons dissociated from ganglia at and below the injury level with behavioral hyperresponsiveness months after SCI suggested that persistent hyperexcitability and SA in nociceptors help to maintain chronic SCI pain [8].

It has been hypothesized that chronic hyperexcitability and SA triggered in nociceptors by SCI represent a pathological induction of a nociceptor hyperfunctional state that in other contexts can compensate for loss of nociceptive function in regions of severe peripheral injury involving partial sensory denervation and

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0304-3959/$36.00
http://dx.doi.org/10.1016/j.pain.2013.06.040

extensive inflammation [71]. If so, mechanisms known to be important for nociceptor sensitization during peripheral injury and inflammation might also be important for enhancing nociceptor activity after SCI. Particularly interesting is transient receptor potential vanilloid-1 (TRPV1), a Ca^{2+}-permeable, nonselective cation channel that is expressed in a large proportion of rodent and human nociceptors [13,35,47,68] and contributes to the activation of pain pathways by heat, acidity, and diverse chemical signals associated with inflammation [14,59,67]. Extensive evidence indicates that upregulation of TRPV1 channels contributes to enhanced pain after peripheral injury and inflammation [9,16,28,36,39,41,50]. Little is known about potential TRPV1 alterations after SCI [22,60], but some evidence suggests that SCI increases TRPV1 expression in spinal cord [20] and DRG [61]. Here we report that SCI increases the sensitivity of primary nociceptors to capsaicin and increases expression of TRPV1 protein in DRG. Moreover, TRPV1 function makes an important contribution to pain-related behavior in this model because chronic behavioral hypersensitivity was reversed by both a TRPV1 antagonist and antisense knockdown of TRPV1 expression. The possibility that enhanced TRPV1 function in primary nociceptors can contribute to pain-related alterations after SCI is supported by our demonstration that SA in DRG neurons is promoted by exposure to extremely low concentrations of a TRPV1 activator after prior SCI.

2. Materials and methods

2.1. Animals

A total of 112 male Sprague-Dawley rats (62 SCI, 22 sham-treated, 28 naïve) were used in this study. All procedures conformed to guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain, and were approved by the Animal Care and Use Committee of the University of Texas Medical School at Houston. Rats (200–300 g) were housed 2 per cage in a controlled environment (12-hour reversed light/dark cycle, 21 ± 1°C) with standard food and water. Animals were acclimated to their cages for at least 1 week before acclimation to the test devices began.

2.2. Spinal cord injury

Rats (200–300 g) were anesthetized with ketamine (80 mg/kg), xylazine (20 mg/kg), and acepromazine (0.75 mg/kg) prior to laminectomy at T10 followed by a spinal contusion using an Infinite Horizon Impactor (150 kDyne, 1-second dwell time; Precision Systems and Instrumentation, LLC, Lexington, KY) [8]. Sham-treated animals received laminectomy and identical treatment except for spinal impact. The surgical site was flushed with saline, overlying muscles were sutured, and the skin incision was stapled with wound clips. The cage containing the rat was placed on a heating pad to maintain temperature at ~37°C for 24 hours. Food and water were placed within easy reach to permit feeding and drinking without assistance. Animals received twice daily intraperitoneal (i.p.) injection of lactated Ringers solution (2 mL) and analgesic (buprenorphine; 0.02 mg/kg, i.p.) for 5 days postinjury and prophylactic antibiotics (2.5 mg/kg; Baytril, Bayer Animal Health, Shawnee Mission, KS, USA) for 10 days. Manual bladder evacuations were performed twice daily until neurogenic bladder voiding returned.

2.3. Behavioral tests

To assess effects of SCI on hind limb motor function, animals were placed in an open field (child's pool) and their spontaneous behavior videotaped and scored on the 21-point Basso, Beattie, Bresnahan (BBB) locomotor rating scale [5]. Animals accepted for this study exhibited BBB scores in the range of 0–1 one day after SCI. Although BBB tests were only performed acutely after injury, informal observation showed that all animals had partial recovery of hind limb motor function 1–2 months after SCI (see also [8]). Behavioral sensitivity data were collected during the animals' active phase in red light and analyzed using blind procedures. Animals received a standard 5-day sequence of tests for hind limb hyperresponsiveness prior to impact and then before and after postinjury manipulations, as described previously [8]. The animal was habituated for 20 minutes in each testing chamber for 3 days, and on days 2 and 3, mechanical and heat test stimuli were given for habituation to the test procedures. Data were collected during identical tests on days 4 and 5. Heat hypersensitivity was tested with the Hargreaves radiant heat method using an IITC Plantar Analgesia Meter (Woodland Hills, CA, USA) to measure the latency to hind paw withdrawal, using a 20-second cutoff to prevent possible tissue injury. Each hind limb was tested, separated by 5 minutes, and the sequence was repeated 3 times, at 20-minute intervals. The 3 latencies were averaged for each hind paw and then the means for both hind paws were averaged for each animal at each test point (pretest, post-SCI, and post-TRPV1 intervention). Mechanical hypersensitivity was tested with a single series of calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA) delivered to the glabrous surface of each hind paw in a sequence determined by the "up-down" method to assess threshold [17].

2.4. Rationale for the use of lumbar DRG neurons and hind limb behavioral measures

Activity in primary nociceptors excites pain pathways and various reflexes, but nociceptor activity does not necessarily lead to pain sensation, especially if pain pathways are interrupted above active nociceptors [71]. This study examined lumbar DRG neurons and hind limb withdrawal reflexes below the spinal injury level for several reasons. First, we wanted to make direct comparisons to results from the vast majority of studies on sensory mechanisms in pain and on TRPV1 function, which have largely utilized lumbar DRG neurons and hind limb reflexes. Second, although hyperreflexia below a spinal lesion may not parallel behavioral alterations above the lesion [4,8], and nociceptor SA at and above a spinal lesion is correlated with enhancement of a supraspinally mediated behavior – vocalization – long after SCI [8], the relationship of nociceptor function to vocalization or other supraspinal responses is highly complex. We started with the flexor reflex most commonly used by pain researchers because it permits well-understood changes in molecular and cellular function to be mapped most directly onto well-defined behavioral consequences. Use of this relatively simple reflex strengthens important conclusions, for example, whether our TRPV1 interventions significantly alter thermal transduction. Third, finding associated alterations of supraspinal responses to simple test stimuli such as heat and von Frey filaments would not be sufficient to demonstrate that altered TRPV1 function drives pain. This will require future demonstration of an associated affective/motivational element using less direct tests, such as operant procedures (eg, [56]). The present study tests whether SCI-induced alteration of TRPV1 function alters nociceptor SA and closely related behavior, establishing in principle whether it might also contribute to SCI pain.

2.5. Dissociation and culture of DRG neurons

After rats were perfused transcardially with cold phosphate-buffered saline under deep anesthesia (75 mg/kg, i.p.; Beuthanasia, Merck Animal Health, Summit, NJ, USA), the thoracic and lumbar segments of the vertebral column were removed, and a laminec-

tomy was performed to expose L4 and L5 DRG. The excised ganglia were minced, and the fragments transferred into Dulbecco's Modified Eagle Medium (Invitrogen, Grand Island, NY, USA) containing collagenase (0.6 mg/mL, Roche, Mannheim, Germany) and trypsin (0.4 mg/mL, Worthington, Lakewood, NJ, USA), and incubated 40 minutes at 34°C. Cells were spun down and plated at low density (see [8]) in 35-mm Petri dishes containing poly-L-lysine (50 mg/mL)-coated cover glass (8 mm, Warner Instruments, Hamden, CT, USA) and then incubated with Dulbecco's Modified Eagle Medium without serum or growth factors at 37°C in 5% CO_2 overnight. After 18–24 hours, living cells were washed and digitally imaged and recorded.

2.6. Whole-cell patch recording from dissociated small DRG neurons

Electrodes with a resistance of ~2 MΩ were pulled from BF150-86-10 glass capillaries (inner diameter 0.86 mm, outer diameter 1.5 mm; Sutter Instrument Co, Novato, CA, USA). Neurons were visualized with differential interference contrast (20×) optics on an inverted microscope (Axiovert 200M; Zeiss, Oberkochen, Germany) and images were acquired with a charge-coupled device (CCD) camera. Neurons (soma diameter ⩽ 30 μm) were recorded in the whole-cell configuration using an EPC-10 amplifier (HEKA Instruments, Lambrecht, Germany). After forming a tight seal (>1 GΩ) the membrane was ruptured. After the whole-cell configuration was established, the cell membrane capacitance and series resistance were electronically compensated. In current-clamp mode, resting membrane potential (RMP) and any SA were recorded. Capsaicin-activated currents were recorded under whole-cell voltage clamp at a holding potential of −60 mV. All experiments were performed at room temperature (~23°C). Signals were filtered at 1 kHz, and digitized at 10 kHz or 2 kHz (for prolonged recordings under current clamp), and acquired using the Pulse software program (HEKA). The pipette solution contained (in mM) 134 KCl, 1.6 $MgCl_2$, 13.2 NaCl, 3 EGTA, 9 HEPES, 1 Mg-ATP, and 0.3 Na-GTP (pH 7.2, 300 mOsM). The bath solution contained (in mM) 140 NaCl, 3 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4 adjusted with NaOH, osmolarity 320 mOsM), and was continuously superfused at 2 mL/min. For perforated patch recordings, the patch pipette was partly filled with the pipette solution by brief immersion, and the remainder of the pipette was then backfilled with pipette solution containing gramicidin. Stock gramicidin solution (50 mg/mL) was prepared in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 100 μg/mL in the pipette.

2.7. In vitro Ca^{2+} imaging in small DRG neurons

Intracellular free calcium, $[Ca^{2+}]_i$, was measured as described previously [78]. Dissociated DRG neurons were incubated in 5 μM Fura 2-AM (Calbiochem, Darmstadt, Germany) in extracellular solution for 20 minutes and then washed 4 times in the dark at room temperature. Fluorescence images were acquired with a Hamamatsu C2400 iCCD (Hamamatsu Corporation, Middlesex, NJ, USA), and measurements of $[Ca^{2+}]_i$ were made with an InCyt Im2 Fluorescence Imaging System (Intracellular Imaging, Cincinnati, OH, USA) equipped with a PixelFly CCD camera (Cooke, Romulus, MI, USA). The ratio of the fluorescence intensity, R (511 nm), was obtained by excitation at 340 nm and 380 nm for each cell of interest.

2.8. Drug treatments

Capsaicin and Fura 2-AM were prepared as stock solutions dissolved in ethanol and DMSO, respectively. AMG9810 was dissolved in DMSO at 1000 times the final concentration and stored as frozen aliquots. Stock solutions were diluted in extracellular or intracellular solution just before use. For in vitro superfusion, solutions were driven by gravity from a series of independent syringes connected to an array of fused silica columns (inner diameter, 200 μm). Rapid exchange of solutions was achieved by shifting the tubes horizontally with a micromanipulator. The distance from the column mouth to the recorded cell was ~100 μm. In vivo delivery of AMG9810 (dissolved in 1% DMSO and 10% TWEEN 80) was achieved by a single i.p. injection (30 mg/kg, 2 mL). Drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO), except Fura 2-AM (Calbiochem).

2.9. Antisense oligodeoxynucleotide knockdown of TRPV1

Previous studies identified an antisense oligodeoxynucleotide (ODN) sequence (ASO sequence) that potently and selectively targets TRPV1 [32] and induces nearly complete degradation of TRPV1 mRNA by RNase H [18]. Moreover, this ASO was taken up well in vivo by DRG neurons after intrathecal (i.t.) delivery and it significantly reduced mechanical hypersensitivity after spinal nerve ligation [18]. We used the most effective ASO sequence reported by these authors, 5′-CATGTCATGACGGTTAGG-3′, and the ineffective mismatch oligodeoxynucleotide (MMO) sequence they used as a control, 5′-CATGCTATGAGCGTTGAG-3′. Both ODNs were purchased from Sigma-Aldrich. One month after SCI, rats were anesthetized with isoflurane, and a chronically indwelling i.t. catheter (PE-10; Becton Dickinson, Sparks, MD, USA) was implanted through a dural slit over the atlantooccipital joint [79]. The catheter terminated at the lumbar enlargement. Rats were housed individually and allowed to recover for at least 5 days before injection. ODNs were injected (45 μg ODN in 5 μL saline, followed by a 10-μL saline flush) during brief isoflurane sedation twice daily for 3 days, and then once per day for 2 days. Tip location and patency were verified postmortem.

2.10. Western blot analysis of TRPV1 protein expression

One month after surgery or 5 days after the knockdown procedure, animals were anesthetized by Beuthanasia and perfused with ice-cold phosphate-buffered saline. Four DRG (bilateral L4 and L5 ganglia) were removed from each rat and immediately placed on dry ice. DRG were homogenized in 300 μL lysis buffer (RIPA; Teknova, Hollister, CA, USA) containing protease inhibitor cocktail (Sigma). After homogenization, samples were sonicated 3 times (10-second pulse), and centrifuged at 14,000 rpm for 10 minutes at 4°C. Protein concentration of lysates was determined by the BCA method (Pierce BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL, USA). Samples were prepared for SDS-PAGE (4–20% Tris-HCl; Bio-Rad, Hercules, CA, USA) by 1:1 dilution with Laemmli buffer and 30 μg of protein was loaded into each well. After electrophoresis, the gel was transferred to a polyvinylidene difluoride membrane and blocked with 10% nonfat dry milk prior to incubation overnight at 4°C with antibody against TRPV1 (Alomone Labs, Jerusalem, Israel), TRPV4 (Enzo Life Sciences, Farmingdale, NY, USA), or β-actin (Abcam, Cambridge, MA, USA). The membrane was incubated with antirabbit or antimouse immunoglobulin G for 1 hour at room temperature, and developed using the ECL kit (Pierce). Protein expression was quantified by optical density using Image J software (National Institutes of Health, Bethesda, MD, USA). Color molecular weight standards were run on each gel. β-actin was used as a loading control.

2.11. Data analysis

Analyses were performed with SigmaPlot 11 (Systat Software, San Jose, CA, USA) and Prism 5.0 (GraphPad, La Jolla, CA, USA). Statistical data are presented as means ± SEM. Comparisons were

made with Student's unpaired *t*-test or 2-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni post hoc tests corrected for multiple comparisons. Paired *t*-tests were utilized for within-animal comparisons. SA incidence was compared using Fisher's exact tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. SCI enhances the responsiveness of isolated nociceptors to capsaicin without increasing the incidence of capsaicin-sensitive neurons

The first question we addressed was whether increased responsiveness to activators of TRPV1 channels occurs after SCI, which might contribute to an SCI-induced hyperfunctional state in nociceptors and consequent pain [71]. We used the best studied activator, capsaicin, which is highly specific for TRPV1 channels [31,55]. To ensure that all available TRPV1 channels in dissociated DRG neurons could be activated, we delivered a saturating dose (3 μM) of capsaicin, and compared capsaicin-evoked currents in voltage-clamped nociceptors dissociated from naïve, sham-operated, and SCI animals. Cells were clamped at −60 mV and tested with a 5-second pulse of capsaicin applied ~100 μm from the soma. Capsaicin-induced current density was not significantly elevated 3 days after injury in neurons dissociated from SCI animals compared to neurons from sham and naïve animals [$F(2,52) = 0.037$; Fig. 1A]. In contrast, neurons dissociated from SCI animals 1–2 months after injury had larger capsaicin responses than neurons from sham-treated and naive groups [$F(2,168) = 9.09$; $P < 0.0001$, Fig. 1B]. When only neurons showing clear responses to capsaicin were included (see also Fig. 1D), the capsaicin responses were distributed evenly across the range of neuron sizes sampled (15–30 μm; Fig. 1C), suggesting that capsaicin sensitivity occurs not only in very small C-fiber neurons, but also in a population of somewhat larger cells that is likely to contain Aδ-fiber neurons as well.

To see if SCI alters the incidence of capsaicin-sensitive neurons, we compared the proportions of dissociated neurons responding with small but reliably detectable inward currents (>40 pA) [20]. No significant differences were found 1–2 months after injury among neurons from SCI, sham-treated, and naive groups in the incidence of capsaicin-sensitive neurons (Fig. 1D). These results suggest that SCI increases the responsiveness of TRPV1-expressing small DRG neurons without increasing the proportion of the neurons expressing TRPV1 (approximately 90% under our conditions).

Because the TRPV1 channel is highly permeable to Ca^{2+} [14], increased responsiveness after SCI should increase Ca^{2+} influx in response to our saturating (3 μM) 5-second capsaicin pulse. Similar to the effect on capsaicin-evoked inward currents, SCI had no significant effect 3 days after injury on capsaicin-evoked increases in $[Ca^{2+}]_i$ in dissociated neurons compared to responses in neurons dissociated from sham-treated and naive animals [$F(2, 37) = 0.88$; Fig. 2A]. In contrast, neurons dissociated from SCI animals 1–2 months after injury exhibited larger capsaicin-induced Ca^{2+} transients than neurons from sham-treated and naive groups [$F(2, 51) = 5.60$; $P = 0.006$, Fig. 2B]. As found with measurements of capsaicin-evoked currents (Fig. 1D), Ca^{2+} imaging measurements showed no change after SCI in the incidence of capsaicin-sensitive neurons, with 84% (16/19) of neurons from naive animals responding to capsaicin, 76% (13/17) from sham-treated animals, and 83% (15/18) from SCI animals. These results provide additional

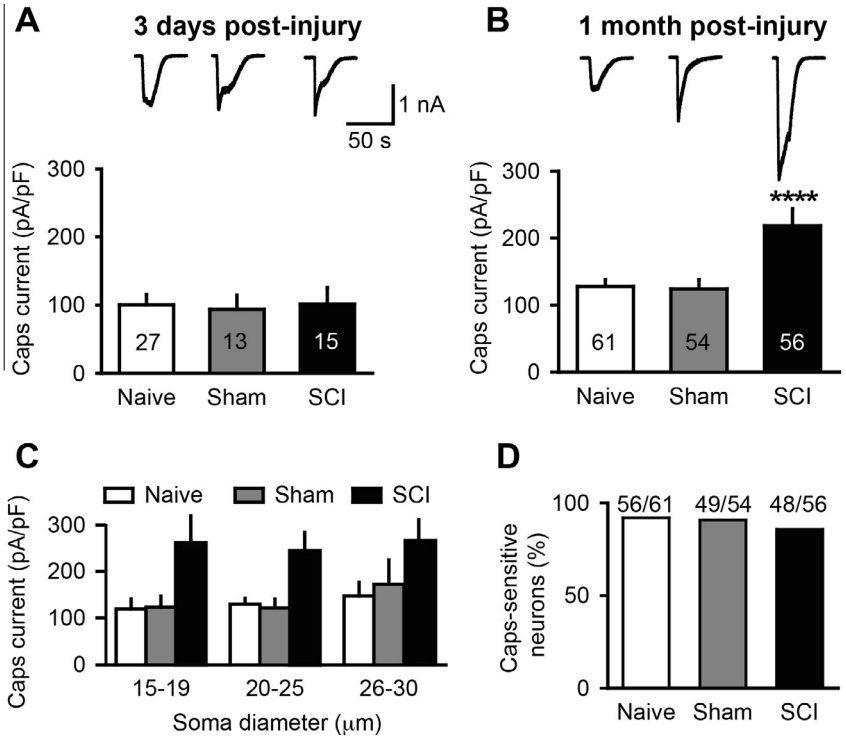

Fig. 1. Spinal cord injury (SCI) enhances capsaicin-evoked currents 1 month after injury without increasing the incidence of small capsaicin-sensitive dorsal root ganglion (DRG) neurons. (A) Lack of an effect of SCI on currents evoked by 3 μM capsaicin 3 days after injury. Typical current recordings are shown above the bar graph. Bars show mean ± SEM of the current densities. Numbers indicate cells tested from each group of animals. (B) Increase in capsaicin currents 1 month after injury. (C) Increased responses after SCI occurs similarly in very small neurons (probably exclusively C-fiber neurons) and cells that are almost medium-sized (probably containing Aδ neurons). (D) Lack of effect of SCI on the incidence of capsaicin-sensitive neurons 1 month after injury. Numbers indicate number of responsive neurons/total number of neurons sampled per group. ****$P < 0.0001$, SCI vs each of the control groups (naive and sham-treated).

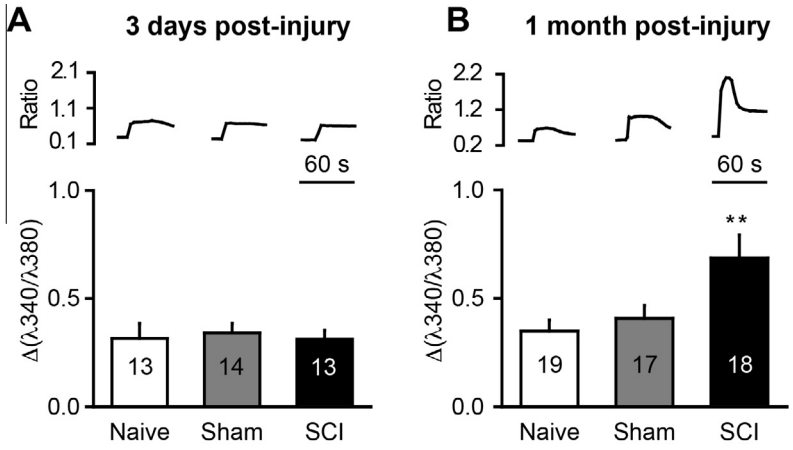

Fig. 2. Spinal cord injury (SCI) enhances capsaicin-evoked Ca^{2+} responses 1 month after injury. (A) Lack of an effect of SCI on Ca^{2+} responses 3 days after injury. Typical Ca^{2+} transients are shown above the bar graph. Numbers indicate cells tested from each group of animals. (B) Increase in Ca^{2+} responses 1 month after injury. $^{**}P < 0.01$, SCI vs each of the control groups (naive and sham-treated).

evidence for SCI-induced enhancement of responsiveness to TRPV1 activators in DRG neurons already expressing TRPV1, and indicate that a consequence of this hyperresponsiveness could be a persistent increase in Ca^{2+} signaling in these nociceptors after SCI.

3.2. SCI increases expression of TRPV1 protein in lumbar DRG

One mechanism that could produce the increases in capsaicin-evoked inward currents and Ca^{2+} transients observed after SCI is increased expression of TRPV1 channels. This possibility is interesting because TRPV1-expressing DRG neurons might respond to the central inflammation caused by SCI like they respond to peripheral inflammation – by upregulating TRPV1 (see Discussion). We thus performed Western blot analysis to determine the expression levels of TRPV1 protein in DRG excised from L4 and L5 ganglia in each group of rats (Fig. 3A). When analyzed 1 month after injury, the amount of TRPV1 protein (relative to β-actin) was significantly different among DRG excised from naive (n = 6), sham-treated (n = 5), and SCI (n = 6) animals [$F(2,16) = 22.93$; $P < 0.0001$], with the levels being higher in ganglia from SCI than sham-treated or naive animals (data not shown). When protein levels in DRG from sham-treated and SCI animals were further normalized to those of DRG from naive animals run on the same gels, levels from SCI animals were significantly greater than those from sham animals (Fig. 3B; $P = 0.006$, paired t-test). These results indicate that at least part of the enhanced sensitivity of small DRG neurons to capsaicin after SCI is caused by increased expression of TRPV1 channels.

3.3. A selective TRPV1 channel blocker reduces SCI-induced behavioral hypersensitivity after SCI

Upregulation of TRPV1 after SCI suggested that TRPV1 channel activity might contribute to the SCI-induced behavioral hypersensitivity we and others have described in the rat spinal contusion model (see Discussion). To begin to test this possibility, we investigated the effects of AMG9810, a specific TRPV1 antagonist [30], on behaviorally expressed hind limb hyperresponsiveness after SCI. Animals received tests of heat and mechanical sensitivity applied to the plantar surface of the hind paws before spinal contusion and then again 4–6 weeks after contusion. Two days later, animals received i.p. injection of either AMG9810 (30 mg/kg) or vehicle, followed 30 minutes later by the same behavioral tests. For heat sensitivity (Fig. 4A), 2-way ANOVA with repeated measures revealed significant effects of the test sequence (pretest, post-SCI, postinjection) [$F(2,17) = 11.37$; $P = 0.007$] and drug treatment [$F(1,11) = 10.70$; $P = 0.008$], with latencies for withdrawal to noxious heat significantly longer after injection of AMG9810 than vehicle ($P = 0.0003$; n = 6 animals in each group). A previous study showed in naive animals that this same i.p. dose of AMG9810 does not significantly alter heat latencies 30 minutes after drug application [56]. For mechanical sensitivity (Fig. 4B), 2-way ANOVA with repeated measures revealed significant effects of the test sequence (injury and recovery) [$F(2,17) = 6.98$; $P = 0.006$] and drug treatment [$F(1,11) = 19.09$; $P = 0.001$], with mechanical thresholds significantly greater after injection of AMG9810 than vehicle ($P = 0.015$; n = 6 animals in each group).

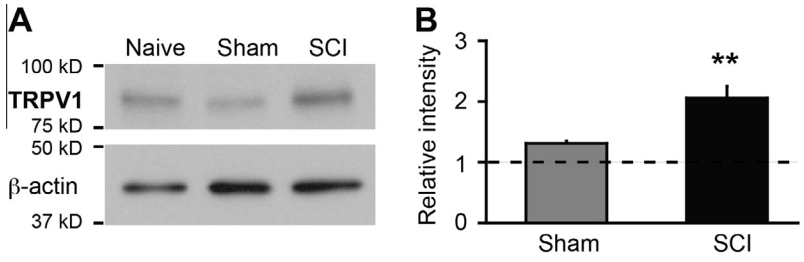

Fig. 3. Spinal cord injury (SCI) increases expression of transient receptor potential V1 (TRPV1) protein in dorsal root ganglia (DRG) excised from L4 and L5 levels 1 month after injury. (A) Example of Western blots. (B) Increase in mean (±SEM) relative optical density of TRPV1 band after normalizing in each case to β-actin and then to the band from a naive animal run on the same gel. $^{**}P < 0.01$, SCI vs sham-treated control group.

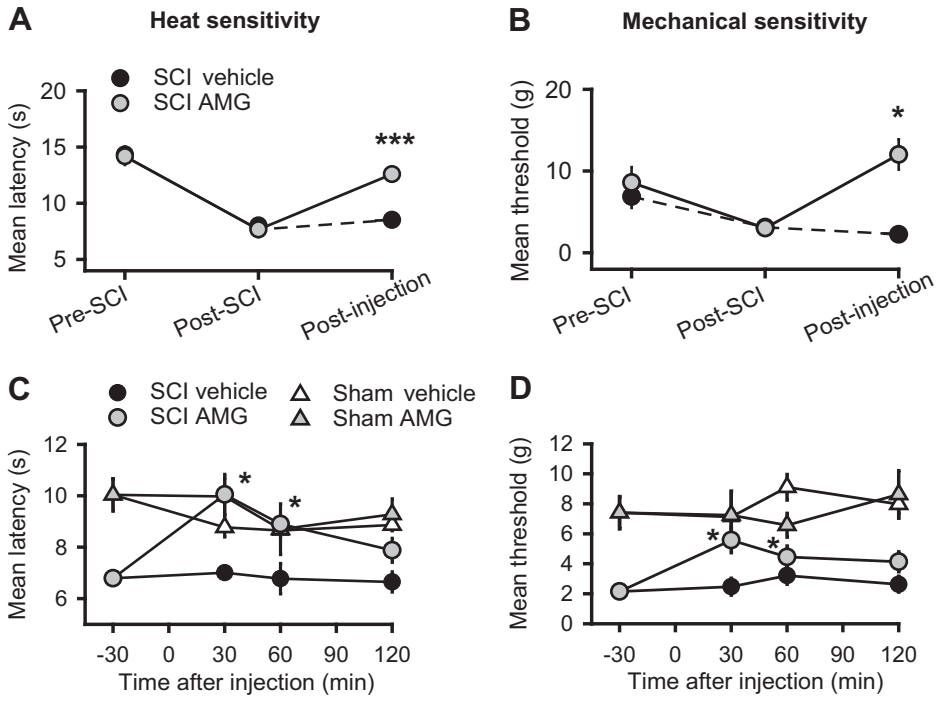

Fig. 4. A specific transient receptor potential V1 (TRPV1) antagonist, AMG9810, reverses behavioral hypersensitivity 4 to 6 weeks after spinal cord injury (SCI). (A) Reversal of hypersensitivity to heat stimulation of the plantar surface of the hindpaw (Hargreaves test) by i.p. injection of AMG9810 (30 mg/kg) 30 min before testing. Hypersensitivity is indicated by the reduced mean (±SEM) latency of the hind limb withdrawal response. The error bars are smaller than the symbols. (B) Reversal of hypersensitivity to weak mechanical stimulation of the same site 30 minutes after AMG9810 injection. (C) Time course of effects of AMG9810 injection on heat sensitivity in SCI animals and sham-treated animals. (D) Time course of AMG910 injection effects on mechanical sensitivity in the same animals. $^{*}P < 0.05$; $^{***}P < 0.001$.

In a separate study (Fig. 4C and D) we examined the time course of the AMG9810 effects after SCI and asked whether sham-treated animals would show any evidence of reflex suppression by the drug. For heat sensitivity (Fig. 4C), 2-way ANOVA with repeated measures revealed a significant treatment effect (SCI vs sham, AMG vs vehicle) [$F(3,10) = 15.24$; $P = 0.0005$]. Partitioning the data into separate treatment groups revealed an across-time effect in the SCI + AMG9810 group [$F(3,9) = 11.85$; $P = 0.0007$] with significant increases in latency compared to baseline at 30 minutes and 60 minutes postinjection ($P = 0.038$ and 0.016, respectively, n = 4). No significant effects were found in the sham group (n = 3) or in the SCI group after vehicle injection, nor were any hints of an effect apparent. For mechanical sensitivity (Fig. 4D), 2-way ANOVA with repeated measures revealed a significant treatment effect (SCI vs sham, AMG vs vehicle) [$F(3,10) = 25.52$; $P < 0.0001$]. Partitioning the data into separate treatment groups revealed an across-time effect in the SCI + AMG9810 group [$F(3,9) = 6.17$; $P = 0.014$] with significant increases in latency compared to baseline at 30 minutes and 60 minutes postinjection ($P = 0.026$ and 0.04, respectively). Again, no significant effects were found in the sham group or in the SCI group after vehicle injection.

These results indicate that ongoing activation of TRPV1 channels contributes substantially to chronic hypersensitivity to heat and mechanical stimuli after SCI. Importantly, the lack of any tendency for AMG9810 injection to increase the latency to respond to heat stimulation in sham-treated animals indicates that, at this dose, AMG9810's behavioral effects are probably not from interference with heat transduction. This conclusion is strengthened by finding that the drug failed to elevate latencies above the pre-SCI levels. Thus, the reversal of heat hypersensitivity by AMG9810 appears to be caused by an effect other than interference with the animals' ability to detect noxious heat.

3.4. Antisense knockdown of TRPV1 expression reverses SCI-induced behavioral hypersensitivity after SCI

To test further the hypothesis that TRPV1 function is important for maintaining behavioral alterations after SCI, we examined the consequences of knocking down expression of TRPV1 protein. Antisense ODNs delivered i.t. have proven to be effective for knocking down proteins expressed in DRG (eg, [1,43,45]). We used an antisense sequence that was previously shown to target TRPV1 selectively and, after i.t. injection in vivo, to be taken up by DRG neurons and reduce behavioral hypersensitivity caused by nerve injury [18,32]. The effectiveness of ASO treatment in knocking down TRPV1 protein expression in our experiments was shown by Western blot analysis on L4 and L5 DRG performed on animals treated for 5 days with ODN injections 4–6 weeks after contusive SCI (Fig. 5A and C). TRPV1 protein levels were significantly decreased (by 63%) after ASO treatment (n = 5) compared to MMO-treated controls (n = 6; $P = 0.0058$). Specificity of the knockdown effect was suggested by the lack of reduction in expression of another TRPV channel, TRPV4 (Fig. 5B and C). The incomplete knockdown of TRPV1 suggests that some channels would have remained to detect noxious heat. A significant decrease in functional expression of TRPV1 channels in primary nociceptors was revealed by a reduction in currents evoked by 3 μM capsaicin after ASO treatment (137 pA/pF, n = 19 neurons from 4 animals) compared to MMO treatment (240 pA/pF, n = 20 neurons from 4 animals; $P = 0.036$) (Fig. 5D). This 43% decrease brought the mean amplitude of capsaicin-evoked currents in ASO-treated SCI animals (Fig. 5D) close to the levels exhibited by neurons taken from naive (128 pA/pF) and sham-treated animals (124 pA/pF) (Fig. 1B). The capsaicin tests may underestimate the degree of functional knockdown because the currents were measured ~36 hours after the last ODN injection, a time when significant recovery of TRPV1 protein

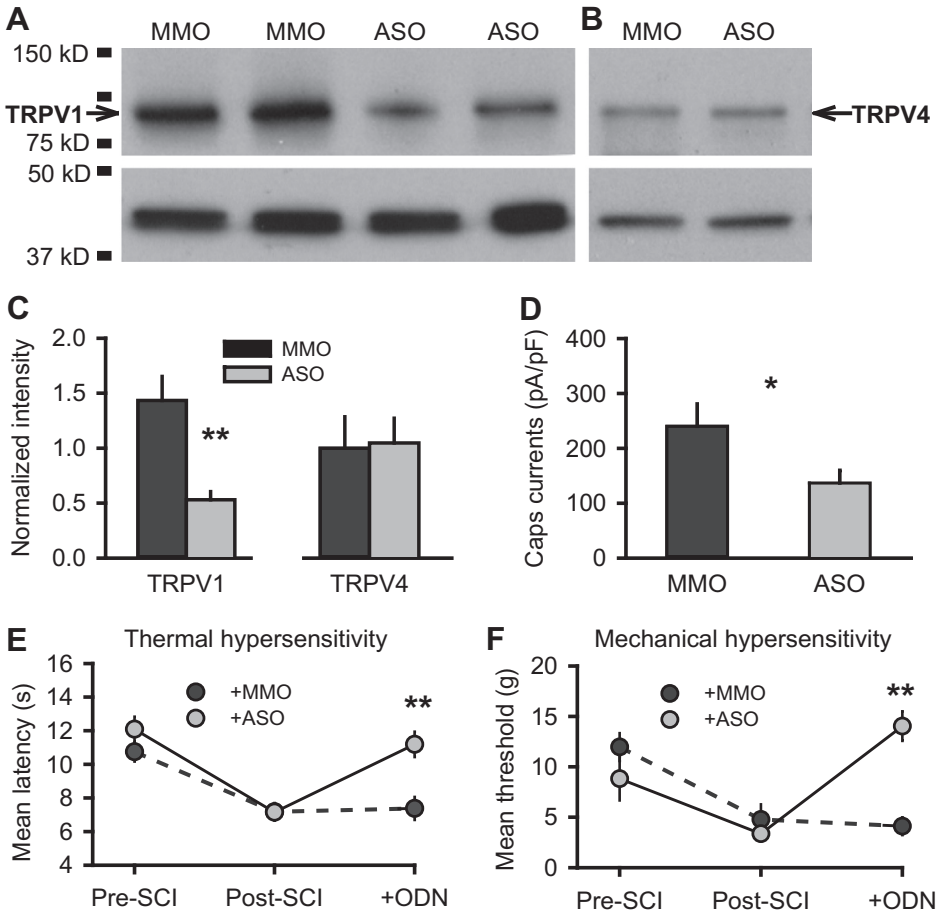

Fig. 5. Antisense knockdown of transient receptor potential V1 (TRPV1) protein reverses behavioral sensitivity 4 to 6 weeks after spinal cord injury (SCI). (A) Representative Western blots illustrating reduction of TRPV1 protein levels after 5 days of intrathecal treatment with an antisense oligodeoxynucleotide (ODN) (ASO) sequence previously shown to specifically target TRPV1. The control was a mismatch ODN (MMO). (B) Examples of TRPV4 blots after TRPV1 ASO and MMO treatments. TRPV4 protein was examined to test specificity of the ASO to TRPV1. (C) ASO treatment compared to MMO treatment knocked down expression of TRPV1 by about 63% (**$P < 0.01$). TRPV4 expression was not decreased. (D) Reduction of currents evoked by 3 μM capsaicin in neurons from ASO-treated animals vs MMO-treated animals 1 day after dissociation (36 hours after the last ODN injection). **$P < 0.01$. (E) Reversal of hypersensitivity to heat stimulation by ASO treatment. (F) Reversal of hypersensitivity to weak mechanical stimulation by ASO treatment. **$P < 0.01$, ASO vs MMO in the postinjection test.

expression should have occurred compared to when samples were taken for Western blot assays (~18 hours after the last ODN injection).

Prior to harvesting the DRG, SCI animals were tested behaviorally ~15 hours after the last ODN injection. At this time, dramatic effects of TRPV1 knockdown were observed on the sensitivity of hind limb withdrawal reflexes. For heat sensitivity (Fig. 5E), 2-way ANOVA with repeated measures revealed a significant effect of the test sequence (pretest, post-SCI, post-ODN) [$F(2,18) = 22.78$; $P < 0.0001$] and ODN treatment [$F(1,9) = 11.03$; $P = 0.0090$], with latencies for withdrawal to moderately noxious heat significantly longer after injection of ASO (n = 6) than MMO (n = 5; $P = 0.0051$). The finding that TRPV1 knockdown did not elevate latencies above the pre-SCI levels shows that the animals' ability to detect noxious heat had not been reduced by the knockdown. For mechanical sensitivity (Fig. 5F), 2-way ANOVA with repeated measures revealed significant effects of the test sequence (injury and recovery) [$F(2,17) = 6.98$; $P = 0.006$]. A partitioned analysis on each factor using 1-way ANOVAs and pairwise comparisons at each test revealed a significant effect of ODN treatment in the post-ODN test, with the threshold for withdrawal higher in ASO-treated than MMO-treated rats ($P = 0.007$). These results show that TRPV1 ASO treatment can effectively reverse chronic behavioral hypersensitivity to heat and mechanical stimuli after SCI.

3.5. Prolonged knockdown but not acute block of TRPV1 channels reduces spontaneous activity in isolated nociceptors

One mechanism by which TRPV1 channels might contribute to chronic behavioral alterations is by promoting SA in nociceptors, which in turn could drive central sensitization, hyperreflexia, and pain [8,12,21,71,77]. This possibility is particularly interesting because ~80% of the small DRG neurons exhibiting SA in vitro after SCI are sensitive to capsaicin [8] (Fig. 1D). Thus, we examined the consequences of TRPV1 knockdown on the high incidence of SA that occurs in dissociated small neurons from L4/L5 DRG long after contusive SCI [8]. Compared to MMO treatment, ASO treatment significantly decreased the proportion of neurons exhibiting SA 4–6 weeks after SCI (Fig. 6A and B; $P = 0.010$, Fisher's exact test). Interestingly, ASO treatment did not reduce the firing rate of the few DRG neurons that did exhibit SA after ASO treatment (Fig. 6C, same neurons as in Fig. 6B). At least 2 general mechanisms might account for the reduction in SA incidence by TRPV1 knockdown. One would be a continuing generation in vitro, 1 day after dissociation, of endogenous TRPV1 activators that drive SA in DRG neurons. An alternative would be a TRPV1-dependent action in vivo that is required for refreshing a long-lasting, intrinsic SA state in small DRG neurons that remains 1 day after isolation of the neurons. Evidence against the first mechanism came from the

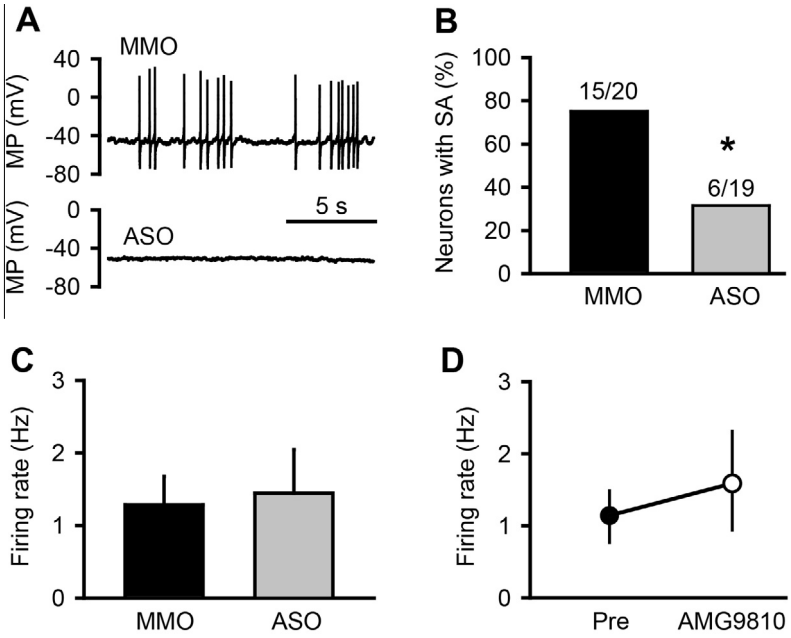

Fig. 6. Spontaneous activity (SA) in dissociated nociceptors is reduced by in vivo knockdown of transient receptor potential V1 (TRPV1) but not by in vitro application of a TRPV1 antagonist. (A) Example of lack of SA in a small dorsal root ganglion (DRG) neuron from an antisense oligodeoxynucleotide (ODN) (ASO)-treated spinal cord injury (SCI) animal (lower trace). A similar neuron from a mismatch ODN (MMO)-treated animal displayed typical SCI-induced SA (upper trace). This and other prolonged recordings were digitized at a low sampling rate (2 KHz), so the action potentials are clipped. (B) Reduction in the incidence of SA in vitro by prior ASO treatment in vivo (ending 36 hours earlier). $^{*}P < 0.05$. (C) Lack of effect of TRPV1 ASO treatment on SA firing rate in the neurons ($n = 6$) that still fired spontaneously after the treatment. (D) Lack of effect of AMG9810 on firing rates of SA neurons (n = 8) dissociated from SCI animals (n = 3).

lack of any inhibitory effect of the TRPV1 antagonist, AMG9810 (10 μM), on SA generated by dissociated small DRG neurons after SCI (Fig. 6D), even though all of the tested neurons responded to capsaicin following washout of the AMG9810 (not shown). This suggests that endogenous ligands acting through the capsaicin binding site [38] are not sufficient to account for the maintenance of SA in dissociated nociceptors after SCI. Even if some TRPV1 ligands are present in vitro (generated within the tested neurons themselves, or released from distant cells in the low-density culture condition), these might not be sufficient to drive SA in the absence of basal activation of TRPV1 by body temperature [29] because the neurons are removed from the body (eliminating synergistic endogenous TRPV1 activators) and tested at 23°C. Instead, the results suggest that continuing activation of TRPV1 channels in vivo is important for refreshing a long-lasting, intrinsic SA state in nociceptors after SCI that probably depends upon alterations of multiple ion channels [8,71,80], and which can sustain SA for at least 1 day after TRPV1 channels become inactive.

3.6. SCI enhances the promotion of SA in isolated nociceptors by very low concentrations of capsaicin

Could the upregulation of TRPV1 channels in DRG after SCI contribute to nociceptor SA when endogenous activators are present? Endogenous activators that excite DRG neurons in vivo after SCI are not yet known. As a first step in addressing this question, we have used low concentrations of capsaicin to ask whether, in principle, an agonist that acts through the capsaicin-binding site on TRPV1 can produce nondesensitizing SA in dissociated nociceptors and, if so, whether this effect is enhanced by SCI. This possibility seemed likely, given that relatively low concentrations of capsaicin can produce ongoing discharge of C-nociceptors (and prolonged pain) at a site of topical application to human skin [46] and that repeated application of low concentrations of capsaicin can produce nondesensitizing activation of C-nociceptors in rats [65]. While most studies using prolonged or repeated capsaicin applications have examined desensitizing excitatory actions produced by relatively high agonist concentrations (eg, [23,24,55,69]), concentrations of capsaicin as low as 30 nM applied to dissociated human DRG neurons can produce sustained discharge during applications as long as 1 minute [6]. To see if even lower concentrations of capsaicin can produce nondesensitizing activation of isolated nociceptors over longer periods, we used perforated patch methods to obtain stable recordings and examined the effects of >10-minute superfusion with 10 nM capsaicin. This is far below the range of concentrations (0.5–5 μM) typically used on dissociated DRG neurons. A typical response to prolonged application of 10 nM capsaicin to an electrically silent, small DRG neuron from a naive animal is illustrated in Fig. 7A1. The capsaicin produced a persistent depolarizing response and sustained repetitive firing without apparent desensitization or action potential accommodation during continuing exposure to the drug. The irregular pattern and rate of firing were indistinguishable from properties of SA observed after SCI [8]. Sustained depolarization during the capsaicin exposure was always accompanied by an increase in amplitude of subthreshold spontaneous oscillations of membrane potential (Fig. 7A2). A minority of neurons from SCI animals were electrically silent, so we compared the incidence of capsaicin-evoked firing in these neurons to that of silent neurons from naive animals. Electrically silent neurons from SCI animals usually responded to 10 nM capsaicin with repetitive firing, showing a significantly greater incidence than did initially silent neurons from naive animals (Fig. 7B; $P = 0.024$, Fisher's exact test). Again, no signs of desensitization or accommodation were observed (data not shown). Furthermore, DRG neurons (n = 16) from SCI animals showed significant depolarization of RMP during the capsaicin exposure (Fig. 7C; $P < 0.001$, paired t-test), and this depolarization was significantly greater than in neurons (n = 16) dissociated from naive animals. Recovery to the baseline RMP after termination of the long pulse of 10 nM capsaicin took somewhat longer in the SCI group than the naive group (138 ± 30 vs 88 ± 14 seconds, respectively), although this difference was not statistically significant. Discharge

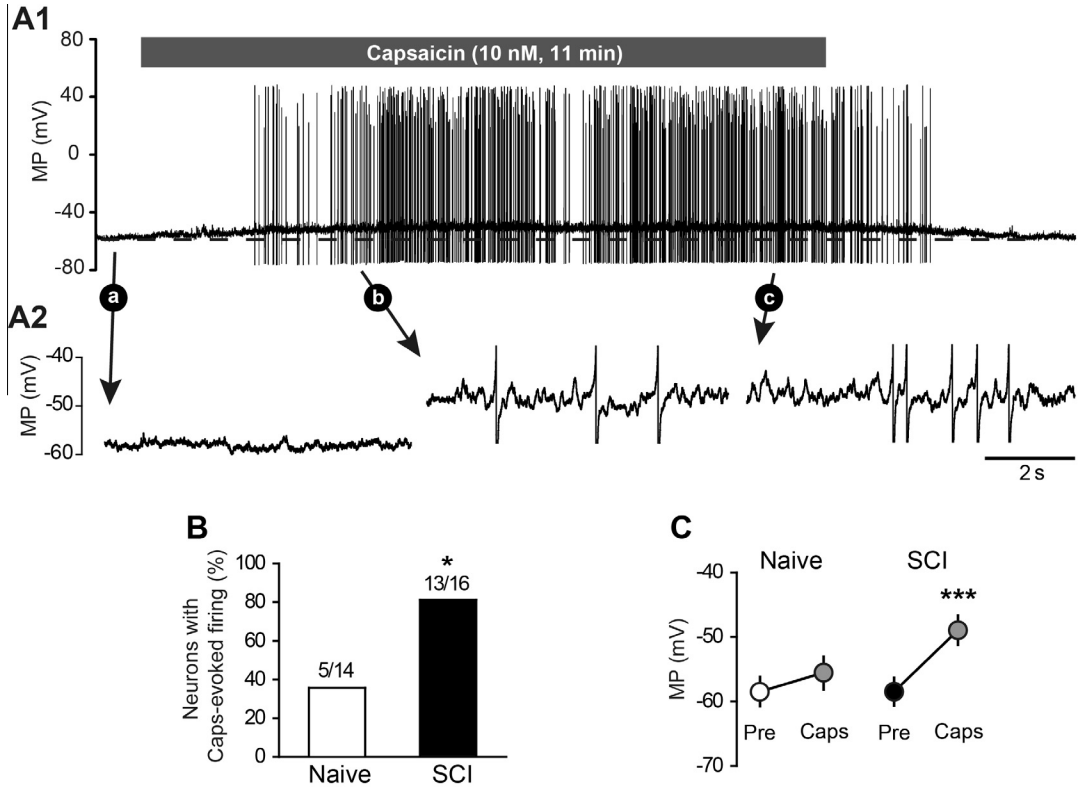

Fig. 7. Spinal cord injury (SCI) enhances the promotion of spontaneous activity (SA) in subsequently dissociated nociceptors by very low concentrations of capsaicin. (A1) Perforated patch recording showing non-desensitizing excitation of a dissociated dorsal root ganglion (DRG) neuron by 11-minute exposure to 10 nM capsaicin. (A2) Expanded traces (a, b, c) from the same record showing the increased amplitude of spontaneous membrane oscillations during the capsaicin-evoked depolarization. Action potentials and afterhyperpolarizations are clipped. (B) Increased incidence of firing evoked by prolonged application of 10 nM capsaicin in neurons dissociated from SCI animals. $^{*}P < 0.05$. (C) Increased depolarization in the same neurons. $^{***}P < 0.001$.

always stopped well before the recovery of RMP. Systematic dose-response relationships were not determined, but we found that 3 nM capsaicin failed to evoke any response in neurons (n = 3) in the naive group that subsequently responded to a high dose of capsaicin (3 μM), whereas 50 nM capsaicin always produced depolarization, sometimes accompanied by discharge in previously silent neurons (3 of 8 neurons from the naive group fired action potentials vs 6 of 10 from the SCI group).

These results show that prolonged activation of TRPV1 channels through the capsaicin-binding site can produce sustained action potential discharge similar to the SA observed after SCI without apparent desensitization or accommodation. This finding provides indirect support for the hypothesis that, after SCI, chronic exposure of nociceptors to low concentrations of endogenous activators of the TRPV1 channel promotes chronic SA and consequent pain-related behavioral alterations.

4. Discussion

We have found that SCI increases the expression of TRPV1 in DRG neurons, enhances the sensitivity of isolated nociceptors to a TRPV1 activator — even at very low concentrations that promote nociceptor discharge without desensitization or accommodation — and that chronic behavioral hypersensitivity induced by SCI is reversed by interventions that reduce TRPV1 function.

4.1. SCI upregulates TRPV1 protein expression and enhances TRPV1 function after SCI

The increase in TRPV1 protein expression in DRG (Fig. 3) extends earlier indications of increased expression of TRPV1 in DRG or the spinal cord after SCI obtained with reverse-transcription polymerase chain reaction [22] and immunohistochemical [61,83] methods. SCI also increased responses to a saturating concentration of capsaicin, as measured by patch-clamp recordings (Fig. 1) and Ca^{2+} imaging (Fig. 2). These findings suggest that SCI increases the number of functional TRPV1 channels in the plasma membrane of primary nociceptors, with some of the increased expression in the cell body. We did not find increased incidence of capsaicin-sensitive neurons after SCI, suggesting that de novo expression was not induced in subpopulations of the small DRG neurons we sampled. Upregulation of TRPV1 in primary afferent neurons has been described in inflammatory conditions [9,16,39,50,81] and in undamaged neurons (likely exposed to inflammatory signals) in neuropathic pain models [28,36,41]. A greater role of inflammation than axon injury in upregulating TRPV1 in L4/L5 nociceptors is suggested by the distance of these DRG from the T10 contusion site (few primary nociceptor axons project that far, so few would be axotomized) (see [8,71]) as well as by extensive spread of inflammation in the spinal cord after SCI [2,11], which could directly impact central processes of primary afferents. Inflammatory signals may also be released from inflammatory cells that infiltrate DRG far from an SCI site [51].

4.2. TRPV1 activation is important for nociceptor SA after SCI

Given in vitro and in vivo evidence for SCI-induced SA being generated chronically in the cell bodies of small DRG neurons [8], an important question is whether upregulation of TRPV1 channels can enhance SA generated in the nociceptor cell body. Endogenous activators of TRPV1 accumulating in DRG after SCI have not been identified, but many endogenous ligands act through the capsaicin-binding pocket in TRPV1 [38,64,72]. We mimicked ongoing actions of this type of activator by prolonged application of capsaicin

at a dose (10 nM) far lower than the EC_{50} values (200–500 nM) reported for dissociated DRG neurons (eg, [52,73]). The low dose of capsaicin caused prolonged firing that showed no desensitization or accommodation for as long as the drug was present (>10 minutes). Stable firing was evoked in 36% of previously silent neurons tested from naive animals and 81% of silent neurons from SCI animals, and this firing (Fig. 7A) was indistinguishable from SA induced by SCI in its irregular pattern and firing rate (see Fig. 6A and [8]). This suggests that upregulation of TRPV1 channels after SCI in vivo can enhance the ability of endogenous TRPV1 activators to promote SA in nociceptors. Capsaicin-induced depolarization was associated with increased amplitude of spontaneous subthreshold oscillations of membrane potential (Fig. 7A2), which are thought to underlie SA in larger DRG neurons [3].

Although direct effects of TRPV1 activators on nociceptors after SCI are likely to be important in vivo, the failure of the TRPV1 antagonist, AMG9810, to suppress SA in vitro suggests that insufficient sources of TRPV1 activation (including heat [29]) are present in our dissociated neuron preparation for this channel to be a major driver of observed SA. This supports our earlier conclusion that SCI-induced SA exhibited by small DRG neurons 1 day after dissociation reflects intrinsic increases in nociceptor excitability [8], and suggests that alterations of additional conductances account for SA in vitro. In contrast, knocking down TRPV1 by 5 days of in vivo ASO treatment dramatically reduced the incidence of SA later observed in vitro (Fig. 6B). This suggests that TRPV1 activity in vivo is required for refreshing a long-lasting, intrinsic SA state in small DRG neurons that remains 1 day after isolation of the neurons. The required in vivo TRPV1 activity might be in several loci, including the nociceptor cell body, other regions of the nociceptor (peripheral or central terminals), other neurons, or other cell types. TRPV1 channels are expressed most abundantly in small DRG neurons that are primarily nociceptors [13,35,47,68], but other TRPV1-expressing neurons have been reported [42,58], including spinal interneurons [44]. Although the functional significance of some of the other TRPV1-expressing neurons is controversial [15,70,82], at least some of the TRPV1-dependent effects we report here might involve TRPV1 channels in neurons other than primary afferent neurons, and TRPV1 channels in nonneuronal cells [25].

4.3. TRPV1 activation is important for behavioral hypersensitivity after SCI

Interventions that decreased TRPV1 function reversed SCI-induced hypersensitivity in behavioral tests (Figs. 4 and 5) that are often used as assays of pain-related behavioral hypersensitivity. A single i.p. injection of the specific antagonist, AMG9810, suppressed both heat hypersensitivity and mechanical hypersensitivity 4–6 weeks after SCI. A qualitatively similar but weaker effect on heat hypersensitivity after spinal contusion at T9 has been reported [60]. However, in that study, <50% of the animals exhibited SCI-induced hypersensitivity to heat, in contrast to this study, where 100% of the SCI animals did. This difference may be due to the systematic habituation to the context and stimuli in our test sequence before data collection, which greatly decreases the variability observed in pain-like behavior after SCI. The decreased hypersensitivity to heat after AMG9810 injection (Fig. 4A) is consistent with our findings indicating a role for upregulated nociceptor TRPV1 channels and increased capsaicin sensitivity of primary nociceptors after SCI. TRPV1 channels are generally thought to be important for detecting noxious heat [13,14,55,59,67] (but see [63,74]). The decreased thermal hypersensitivity observed in the presence of AMG9810 did not result from failure to detect the heat stimulus because the tested SCI animals still responded to heat after drug injection (with response latencies like those of naive animals). This observation and demonstrations that the same dose of AMG9810 had little effect on latency to withdraw to heat stimulation in sham-treated (Fig. 4C) or naive animals [56] suggest that this dose of AMG9810 primarily blocks TRPV1-dependent sensitization mechanisms rather than heat transduction, which is consistent with other evidence in rodents for complex contributions of TRPV1 to sensory transduction and sensitization [10,74,84]. Nearly identical reversal of heat hypersensitivity without elimination of heat responses (Fig. 5D) was found after repeated i.t. injection of an antisense ODN that knocked down TRPV1 protein expression and function (Fig. 5A–C). Together, these findings suggest that enhanced TRPV1 function is important for maintaining heat hypersensitivity below the injury level after SCI.

Both AMG9810 and TRPV1 antisense ODN treatment also reversed mechanical hypersensitivity. This finding is interesting because, while TRPV1 channels may exhibit mechanosensitivity under some conditions [37,40], they do not contribute to activation of primary nociceptors by the punctate cutaneous test stimuli we used, and that are typically used to assess mechanical allodynia [10,48,54]. Instead, TRPV1 activity is likely to be required after SCI for maintaining central sensitization in the spinal cord that amplifies behavioral responses to mechanical and other stimuli transduced by diverse sensory receptors [75]. Central sensitization after SCI might involve spinal TRPV1, both in central terminals of primary nociceptors where it produces presynaptic facilitation [19] and in interneurons where it may disinhibit pain-related activity [44]. Our findings suggest that TRPV1 in nociceptors promotes nociceptor SA to drive central sensitization. The reversal of behavioral hypersensitivity by a dose of AMG9810 that does not block heat transduction suggests that TRPV1 blockers might be effective clinically at doses that reduce nociceptor SA and central sensitization without compromising detection of dangerously hot stimuli. This observation plus the recent development of new TRPV1 blockers that fail to produce unwanted hyperthermia [62] suggest that low doses of newer TRPV1 blockers might be used clinically to reduce pain maintained by ongoing TRPV1 activity without producing these 2 adverse side effects that have been problematic in humans [31,55].

Central sensitization driven by nociceptor SA may recurrently refresh the SA state in nociceptors, which can persist for at least 1 day in the absence of continuing TRPV1 activation. The possibility of a TRPV1-dependent positive feedback loop between nociceptor SA and central sensitization [71] is supported by the joint suppression of sensitized behavioral responses (Fig. 5) and nociceptor SA by antisense knockdown of TRPV1 (Fig. 6A, B). A critical role for SA in primary nociceptors for driving central sensitization and pain-related behavior after SCI is indicated by our finding [80] that nociceptor SA and behavioral hypersensitivity to heat and mechanical stimulation are also suppressed by antisense knockdown of a Na^+ channel, Nav1.8, that is primarily expressed in nociceptors. These results indicate that sensitizing SA in nociceptors depends in part upon TRPV1 function that is persistently enhanced by SCI, and point to TRPV1 as a potential target to treat chronic SCI pain.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by grants from the Craig H. Neilsen Foundation and the Department of Defense United States Army Medical Research Acquisition Activity (USAMRAA). We thank Julia Hadden for technical assistance and Drs. Carmen Dessauer, Jeffrey Frost, Raymond Grill, and Hongzhen Hu for helpful suggestions.

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Submitted to Experimental Neurology 10/01/13

Review

Neuroinflammatory contributions to pain after SCI: roles for central glial mechanisms and peripheral host defense

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Keywords: Neuropathic pain
Microglia
Astrocytes
Nociceptors
Innate immune system

ABSTRACT

Neuropathic pain after spinal cord injury (SCI) is common, often intractable, and can be tragically debilitating. During the last decade studies of animal models have shown that both central neuroinflammation and behavioral hypersensitivity (indirect reflex measures of pain) persist chronically after SCI. Interventions that reduce neuroinflammation have been found to ameliorate pain-related behavior, including treatment with agents that inhibit activation states of microglia and/or astroglia (including IL-10, minocycline, etanercept, propentofylline, ibudilast, licofelone, SP600125, carbenoxolone). Reversal of pain-related behavior has also been shown with disruption by an inhibitor (CR8) and/or genetic deletion of cell cycle-related proteins, deletion of a truncated receptor (trkB.T1) for brain-derived neurotrophic factor (BDNF), or reduction by antisense knockdown or an inhibitor (AMG9810) of the activity of channels (TRPV1 or Nav1.8) important for electrical activity in primary nociceptors. Nociceptor activity is known to drive central neuroinflammation in peripheral injury models, and nociceptors appear to be an integral component of host defense. Thus, emerging results suggest that spinal and systemic effects of SCI can activate nociceptor-mediated host defense responses that interact via neuroinflammatory signaling with complex central consequences of SCI to drive chronic pain. This broader view of SCI-induced neuroinflammation suggests new targets, and additional complications, for efforts to develop effective treatments for neuropathic SCI pain.

Contents

Introduction

While everyday experience makes the association between inflammation and pain obvious, it is only within the last decade that investigators have linked neuroinflammatory consequences of SCI to the intractable pain that many SCI patients endure. The realization that widespread neuroinflammation plays a major role in driving neuropathic pain after SCI has been accompanied by discoveries of unexpected overlap of neuroinflammatory mechanisms that drive persistent pain after injury to the central nervous system and after injury to peripheral tissues. This review discusses experimental evidence and a new conceptual perspective that provide insight into how neuroinflammation contributes to SCI pain. Although progress in developing effective treatments for neuropathic SCI pain has been slow, a number of recent preclinical findings suggest novel therapeutic targets that may offer promise as additional treatment options.

Prevalence, types, and properties of pain after SCI

Estimates of its prevalence vary greatly, but it is likely that chronic pain afflicts a majority of SCI patients (Dijkers et al., 2009). This pain is divided into two general classes (Bryce et al., 2012). **Nociceptive pain** is defined as pain caused by activity generated by normal mechanisms in the peripheral terminals of primary nociceptors. Nociceptive pain after SCI is caused by common sequelae of SCI (Finnerup and Baastrup, 2012), including overuse of the upper body, muscle weakness, poor posture, spasticity, and other problems of the musculoskeletal system. Nociceptive visceral pain after SCI can be caused by constipation, nociceptive cutaneous pain from pressure sores, and nociceptive headache from autonomic dysreflexia.

In contrast, **neuropathic pain** is defined as resulting directly from damage to or disease of the nervous system (Jensen et al., 2011). Neuropathic pain caused by SCI, like other forms of neuropathic pain (Costigan et al., 2009), is considered a purely pathological, maladaptive consequence of damage to the nervous system (Gwak and Hulsebosch, 2011; Walters, 2012). Neuropathic pain after SCI occurs in 40-50% of patients, it is often permanent and intractable to available treatments, and it is sometimes the most debilitating result of SCI (Finnerup, 2012). Neuropathic SCI pain is subdivided into at-level and below-level pain, felt at and below the level of the spinal injury (Bryce et al., 2012). Both types of neuropathic pain summon descriptors such as hot-burning, sharp, shooting, electric shock-like, tingling, squeezing, painfully cold, pricking, and/or pins and needles. This pain often occurs spontaneously and can be evoked by stimuli

that either are not normally painful (allodynia), or in exaggerated form by noxious stimuli (hyperalgesia). Because of its frequent severity and resistance to treatment, neuropathic pain after SCI has received far more experimental study than has nociceptive pain. However, as will be discussed below, chronic nociceptive pain and neuropathic pain after SCI probably involve shared neuroinflammatory mechanisms.

The use of animal models to study mechanisms of neuropathic SCI pain

As mentioned in other articles in this special issue, SCI is always followed by neuroinflammation. Clinical observations, including chronic elevation of proinflammatory cytokines in the CSF and blood of patients (Davies et al., 2007; Kwon et al., 2010; Stein et al., 2013), are consistent with the possibility that neuroinflammation that may promote pain persists long after SCI, but mechanistic studies in patients have not been performed. The most compelling evidence for neuroinflammatory contributions to neuropathic pain after SCI has come from animal studies, primarily in rodents. The general strategy in these studies has been to explore the behavioral and cellular effects of an SCI produced by one of several, relatively standard procedures, usually applied to a thoracic segment but sometimes to a cervical or upper lumbar segment. These include contusion caused by impact on the exposed dural surface of the cord, brief compression of the cord by a clip, surgical hemisection, discrete lesion (often electrolytic) of the anterolateral tract, localized excitotoxicity produced by injection of a glutamate receptor agonist, or dorsal root avulsion that damages the dorsal horn (Christensen et al., 1996; Detloff et al., 2013; Hulsebosch et al., 2009; Onifer et al., 2007; Siddall et al., 1995; Vierck et al., 2000; Wieseler et al., 2010; Yezierski et al., 1998; Young, 2002). The most common index of pain in animal studies has been an increase in cutaneous mechanical sensitivity measured as a decrease in the bending force required to elicit a behavioral response by a series of "von Frey" filaments of varying stiffness, usually applied to the plantar surface of a hindpaw. Another common index of pain is an increase in heat sensitivity measured as a decrease in latency to withdraw to a radiant stimulus applied to the same site (Hargreaves test). A limitation of these commonly used tests is that the measured responses are spinally mediated and may not reveal anything about crucial emotional and cognitive aspects of pain, which appear to be cortically mediated (e.g., Mendell, 2011; Wiech et al., 2008). This problem is especially severe when below-level reflex measures are used, such as the pervasive hindlimb "mechanical allodynia" and "thermal hyperalgesia" tests performed with von Frey hairs and radiant heat, because even moderately severe SCI may cause extensive interruption of ascending tracts (Baastrup et al., 2010; Detloff et al., 2008). Better indices of pain involve

behaviors that require supraspinal processing, such as vocalization, licking, guarding, and facial grimacing (Baastrup et al., 2010; Bedi et al., 2010; Sotocinal et al., 2011; Yezierski and Vierck, 2010). Perhaps the most promising index of a pain-like emotional state is offered by the conditioned place preference (CPP) test, an operant measure that reveals the cognitively accessible aversiveness of an ongoing state in an animal (Navratilova et al., 2013; Sufka, 1994). The CPP test has implicated ongoing pain in peripheral neuropathic and inflammatory pain models (King et al., 2009; Okun et al., 2011) by showing that a rat will choose to spend time in a chamber that had previously been paired with injection of an effective analgesic. So far, only a single publication has described the use of CPP to reveal an ongoing pain-like state after SCI; rats that received an electrolytic lesion in the ventrolateral quadrant of the cervical cord showed not only long-lasting mechanical hypersensitivity, but preferred a chamber that had been paired a month after SCI either with intraventricular injection of the alpha adrenergic agonist clonidine or with electrical stimulation of motor cortex (Davoody et al., 2011). It will be important to see if CPP is produced by interventions that target neuroinflammatory mechanisms, and whether such CPP occurs in other models of SCI. Preliminary results have revealed significant CPP in a rat spinal contusion injury model (Yang, Q., Hadden, J., Crook, R.J., Zebda, D., Walters, E.T., unpublished observations).

Types of mechanisms implicated in neuropathic SCI pain

Neuroinflammatory mechanisms are just one set of mechanisms driving SCI pain, and will be reviewed in the following sections. Other general mechanisms have also been proposed, and these may operate independently or synergistically with neuroinflammation to promote SCI pain. Some of these represent direct effects of injury on spinal tissue. An immediate effect of spinal injury is intense activation of nearby neurons by depolarizing agents released during the injury (e.g., K+ and glutamate from dying and injured cells) (Finnerup and Jensen, 2004; Yezierski, 2009). Although direct, injury-induced excitation of pain pathways should wane with time after SCI, the intense early activity should be propagated widely in the CNS and in different parts of pain pathways it might induce long-term synaptic alterations similar to those underlying long-term memory in the brain, such as late-phase long-term synaptic potentiation (Asiedu et al., 2011; Laferriere et al., 2011; Marchand et al., 2011; Rahn et al., 2013). Indeed, memory-like alterations have been implicated in spinal neurons that may maintain chronic central sensitization and consequent pain (Crown et al., 2006; Tan and Waxman, 2012). Intense activation of neural pathways during SCI might also trigger long-term changes in ion channel

function and increases in intrinsic excitability of neurons in pain pathways, for example by increasing the expression in neurons of voltage-gated Na^+ channel subunits (Hains et al., 2003; Hains et al., 2005) or expression of the $\alpha 2$-$\delta 1$ voltage-gated Ca^{2+} channel subunit (Boroujerdi et al., 2011). Other direct effects of SCI are anatomical, including the transection or demyelination of descending and ascending axons in the cord. Below-level pain after partial interruption of axonal tracts has been attributed to a loss of descending inhibition onto below-level nociceptive pathways so that activity in surviving ascending pain pathways is persistently increased (Bruce et al., 2002; Hains et al., 2002; You et al., 2008). Below-level behavioral hypersensitivity has also been associated with reduced activity in local GABAergic inhibitory interneurons in the spinal dorsal horn (Drew et al., 2004; Gwak et al., 2006), and with an apparent apoptotic loss of GABAergic neurons in lumbar segments relatively distant from a contusion injury at T11 (Meisner et al., 2010). Interruption of ascending pain pathways might also promote neuropathic pain; for example, deafferentation by SCI may cause increased sensitivity of pain-related neurons in the thalamus to other inputs (Wang and Thompson, 2008) and activity-dependent reorganization of cortical pain networks (Nardone et al., 2013). SCI-induced changes in the balance of excitatory and inhibitory influences at any level of the pain pathway may result in neuropathic pain (Finnerup and Jensen, 2004; Whitt et al., 2013). Anatomical changes in the spinal cord that might promote neuropathic pain include the sprouting of primary nociceptors (Krenz and Weaver, 1998; Ondarza et al., 2003). Interestingly, SCI triggers an enhanced, intrinsic growth state in primary nociceptors (Bareiss et al., 2013; Bedi et al., 2012), as well as other alterations in these sensory neurons (see below). Another anatomical alteration caused by SCI is disruption of the blood-spinal cord barrier (BSCB) in the region of injury (Popovich et al., 1996). Disruption of the BSCB has been associated with pain after peripheral injury or inflammation (Beggs et al., 2010; Brooks et al., 2005), perispinal inflammation (Tenorio et al., 2013), and SCI (Lin et al., 2012). Among other effects, disruption of the BSCB will permit blood-borne myeloid and lymphoid immune cells to enter the spinal cord parenchyma and exert direct inflammatory actions on central neurons and glia (Skaper et al., 2012; Zhang et al., 2011) (Fig.1).

Neuroinflammatory mechanisms of neuropathic SCI pain

Why spinal neuroinflammation should produce neuropathic SCI pain

Neuroinflammation is commonly and simply defined as inflammation of part of the nervous system, while inflammation has been defined immunologically as a response of the innate immune system that combats infection both by recruiting cells and proteins to destroy pathogens, and by increasing the flow of lymph containing pathogens and antigen-presenting cells to lymphoid tissues in order to initiate the more delayed adaptive immune response (Murphy, 2012). Many investigators would add that inflammation also functions to repair injured tissue, including neurons (Benowitz and Popovich, 2011). Cells of the innate immune system are thus considered important for neuroinflammation (Donnelly and Popovich, 2008), especially cells in the myeloid lineage, including macrophages, granulocytes, dendritic cells, and mast cells. Particular emphasis has been placed by investigators of pain on the myeloid cells residing in the CNS parenchyma that are closely related to macrophages – microglia (Calvo and Bennett, 2012; Graeber and Christie, 2012; Schomberg and Olson, 2012; Taves et al., 2013). However, inflammation involves responses to pathogens and tissue damage that are mediated by interactions of myeloid immune cells with various other cell types, including endothelial cells and neurons. Non-myeloid cells in the CNS contributing to neuroinflammation that have received considerable attention in pain studies are astrocytes. Indeed, interlinked, overlapping functions and dysfunctions ("gliopathy") of astroglia and microglia appear to be especially important for neuropathic pain (Hulsebosch, 2008; Ji et al., 2013; McMahon and Malcangio, 2009; Watkins et al., 2001).

Four general observations suggest that SCI-induced neuroinflammation should produce pain. First, spinal inflammatory reactions induced by peripheral injury and/or inflammation produce pain-related behavior. Pain associated with spinal neuroinflammation (activation, migration, and proliferation of microglia and astrocytes) was first observed after peripheral injury and inflammation (reviewed by DeLeo and Yezierski, 2001; Watkins et al., 2001; Ji et al., 2013). Peripheral nerve injury was later found to promote central neuroinflammation by triggering infiltration of leukocytes into the cord (Skaper et al., 2012; Sweitzer et al., 2002) probably by enabling disruption of the BSCB (Brooks et al., 2005; Gordh et al., 2006; Huber et al., 2001). Second, SCI causes the generation and widespread release of proinflammatory cytokines (e.g., Alexander and Popovich, 2009) and changes in the levels of some of these, such as IL-6, have been correlated with behavioral indicators of pain (Detloff et al., 2008). Third, in uninjured

animals intrathecal injection of proinflammatory cytokines, including IL-1β and IL-6, produces reflex hypersensitivity (DeLeo et al., 1996; Reeve et al., 2000). Fourth, increasing evidence indicates that neuroinflammation after SCI, like neuropathic SCI pain, persists indefinitely (Beck et al., 2010; Byrnes et al., 2011; Dulin et al., 2013a; Fleming et al., 2006; Nesic et al., 2005). Specific mechanisms that have been linked to neuropathic SCI pain are reviewed below, separated into mechanisms that have been studied primarily in microglia and primarily in astrocytes. Bear in mind, however, that some of these mechanisms may occur in both types of glia, and many probably represent reactions that are promoted in one cell type by signaling from the other glial cell type (and from non-glial cells). Figure 1 depicts some of the complex extracellular signaling among microglia, astrocytes, and neurons that may be important for neuropathic SCI pain. Given the general arguments just listed for why SCI-induced neuroinflammation should produce pain, a puzzle is why a substantial fraction of SCI patients with injuries indistinguishable from other SCI patients fails to report neuropathic pain (Dijkers et al., 2009). These dramatic differences within the SCI patient population in the severity of neuropathic pain emphasize the complexity of the systems involved. One intriguing factor could be differential operation of endogenous pain suppression mechanisms, which may be recruited more effectively in some SCI patients than others. If so, identification of such suppressive mechanisms might lead eventually to clinical applications for SCI pain.

SCI pain mechanisms associated with microglia

The earliest direct evidence in animal models that inflammatory responses contribute to SCI pain came from the ability of interventions that reduce signaling by cells in the innate immune system, such as microglia and macrophages, to reduce reflex hypersensitivity after SCI. IL-10 is a potent anti-inflammatory cytokine that reduces the activation of many immune cells, including macrophages, microglia, and astrocytes, and is also produced by glia (Graeber and Christie, 2012; Thompson et al., 2013). Systemic injection of IL-10 shortly after excitotoxic spinal injury delayed the onset of and decreased the amount of excessive grooming caused by this type of injury, associated with a reduction both in neural damage and in signs of neuroinflammation (including reduced spinal expression of IL-1β, COX-2, and iNOS) (Plunkett et al., 2001). Moreover, knockout of IL-10 accelerated the onset of pain-related behavior and expansion of the lesion (Abraham et al., 2004). Recently, IL-10 delivered by a herpes simplex virus (HSV) vector after spinal contusion injury decreased spinal TNFα expression and astrocyte activation assessed by glial fibrillary acidic protein (GFAP) immunochemistry (see

below). Hindlimb mechanical and heat hypersensitivity were reduced by the IL-10 delivery and, importantly, so was an operant measure of evoked pain (Lau et al., 2012). These pain suppressive effects are consistent with direct actions of IL-10 on activated microglia, although many other cell types may have contributed to IL-10's effects in these studies.

Widespread activation of microglia (spinal and supraspinal) occurs after SCI, as indicated by a shift in morphology from a ramified to ameboid shape and upregulation of the microglial markers CD11b/c/CCR3 (OX-42), major histocompability complex II (MHC II), or ionized calcium-binding adaptor molecule-1 (Iba-1) (e.g., Popovich et al., 1997; Watanabe et al., 1999; Dijkstra et al., 2000; Nesic et al., 2005; Schwab et al., 2005; Detloff et al., 2008). The concept of glial activation or reactivity probably involves multiple states and functions in microglia and other myeloid cells (Hawthorne and Popovich, 2011), but these complications have largely been ignored thus far in studies of microglial contributions to SCI pain. Proliferation and migration of microglia also occur (Byrnes and Faden, 2007; Zai and Wrathall, 2005). Because of the prominent roles of macrophages in peripheral inflammation, and of the many proinflammatory signals secreted by both macrophages and microglia on other examples of pain-related behavior (Ellis and Bennett, 2013; Kettenmann et al., 2011; Ramesh et al., 2013), microglia were prime candidates to drive SCI pain. This possibility was strongly supported by the demonstration that spinal injection of microglia that had been activated by ATP was sufficient to produce mechanical hypersensitivity in uninjured animals (Coull et al., 2005).

Contributions of microglia to neuropathic pain after SCI have largely been studied using the tetracycline antibiotic, minocycline, to inhibit microglial activation. Although minocycline remains an effective and popular inhibitor of microglia, it has many other reported effects (Garrido-Mesa et al., 2013), which limit the conclusions that can be drawn about how it may reduce SCI pain. These include other actions that could also inhibit pain-related mechanisms, such as inhibition of matrix metalloproteinases (Matsumoto et al., 2009), scavenging of free radicals (Ulgen et al., 2011), inhibition of poly(ADP-ribose) polymerase-1 (PARP-1) (Alano et al., 2006), and inhibition of voltage-gated Na^+ channels (Kim et al., 2011). The first use of minocycline and the first explicit test of microglial involvement in SCI pain was reported less than a decade ago by Hains and Waxman (2006). One month after thoracic contusive SCI, intrathecal infusion of minocycline reduced signs of microglial activation (decreasing P-p38 MAPK in OX-42-positive cells and reducing the number of OX-42-positive cells with an activated morphology) (see also Crown et al., 2008), reduced spontaneous and evoked activity in lumbar dorsal horn neurons, completely reversed heat hypersensitivity of hindlimb reflexes, and largely reversed mechanical hypersensitivity of the same reflexes. These investigators then provided evidence that microglia

contribute to SCI-induced reflex hypersensitivity by an ERK1/2-dependent release of PGE2 and probable binding to neuronal EP2 receptors in the lumbar dorsal horn (Zhao et al., 2007b), and a recent study found similar effects of SCI on P-p38 MAPK and PGE2 production that could be ameliorated by acupuncture (Choi et al., 2012). Zhao et al. (2007) showed a robust suppression of SCI-induced behavioral hypersensitivity by intrathecal injection of an EP2 receptor antagonist, while an earlier study had shown that systemic application of a COX-2 inhibitor reduced mechanical and heat hypersensitivity in hindlimbs and forelimbs associated with decreased PGE2 levels in the cord after contusive SCI (Hains et al., 2001). However, the potential clinical significance of the COX-2/PGE2 findings can be questioned because nonsteroidal anti-inflammatory drugs that primarily inhibit COX activity are not very effective in relieving neuropathic pain after SCI in humans (Cardenas and Jensen, 2006). On the other hand, neuroinflammation is associated with elevated arachidonic acid (AA) metabolism, and AA is metabolized not only to prostaglandins by COX but also to leukotrienes by 5-lipoxygenase (LOX). Interestingly, leukotrienes, like prostaglandins, contribute to behavioral hypersensitivity in other pain models (Noguchi and Okubo, 2011). A recent study (Dulin et al., 2013a) showed that both the proinflammatory leukotriene B4 and PGE2 were elevated at a spinal contusion site 9 months after injury, and that treatment of rats for 1 month starting 8 months after SCI with a dual COX/5-LOX inhibitor, licofelone, reversed mechanical hypersensitivity of the hindpaws. If licofelone is also found to reverse operant measures of pain in rodents, it would be one of the more promising potential treatments for neuropathic SCI pain to be suggested by preclinical studies.

Evidence for supraspinal microglial contributions to neuropathic SCI pain have also been found. Spinal contusion injury increased OX-42 expression in cells in the ventral posterolateral (VPL) nucleus in the thalamus (see also Detloff et al., 2008) and increased neuronal levels of a chemokine, CCL21, in the thalamus and spinal cord (Zhao et al., 2007a) (see also Wu et al., 2013a). Electrical stimulation of the spinothalamic tract also increased CCL21 in the thalamus. Intra-VPL injection of CCL21 increased microglial activation and increased hindlimb mechanical and heat sensitivity, whereas both a neutralizing antibody to CCL21 and minocycline reversed the SCI-induced behavioral hypersensitivity as well as the microglial and neuronal effects produced in the VPL (Zhao et al., 2007a). These results suggest that pain-promoting activation of supraspinal neurons is driven by activity-dependent release of at least one chemokine, CCL21, from spinothalamic tract neurons after SCI.

Some SCI studies differ from those just described in suggesting that microglial activity is more important for the early induction than the later maintenance of neuropathic pain, as was

previously indicated by studies using peripheral neuropathic pain models (Ledeboer et al., 2005; Owolabi and Saab, 2006; Raghavendra et al., 2003; Zhuang et al., 2005). Early administration of minocycline (beginning minutes after injury and repeated for 2-5 days afterwards) attenuated the development of mechanical and heat sensitivity as shown by reflex tests 1 and 2 weeks after spinal hemisection (Marchand et al., 2009) or tests 4 weeks after spinal contusion (Tan et al., 2009). Thus, early administration of minocycline, unlike later treatments (Hains and Waxman, 2006; Zhao et al., 2007b), produced reductions in behavioral hypersensitivity that long outlasted the drug application. TNFα, a proinflammatory cytokine that is synthesized and released by various cell types, including neurons and astrocytes, but in the CNS primarily by microglia (Kraft et al., 2009), plays a particularly important role in the initiation of inflammatory cascades (Montgomery and Bowers, 2012). Interestingly, etanercept, a fusion protein blocker of TNFα, dramatically reduced behavioral hypersensitivity 1 to 4 weeks after spinal hemisection if given for 2 days beginning immediately after injury, but had no effect when delivered 2 to 3 weeks after injury (Marchand et al., 2009). An interesting possibility, consistent with the long-lasting activation of astrocytes after SCI (see next section), is that early signaling by reactive microglia promotes a longer-lasting activation of astrocytes that helps to maintain chronic SCI pain.

SCI pain mechanisms associated with astrocytes

Although derived from neuroepithelial rather than the myeloid or lymphoid progenitors of immune cells, astrocytes are essential participants in neuroinflammation, which depends upon interactions among astrocytes, microglia (and sometimes other host defense cells), and neurons (Alexander and Popovich, 2009). Indeed, many investigators consider astrocytes to be part of the innate immune system (Ransohoff and Brown, 2012). Numerous studies have indicated that astrocytes make major contributions to pain-related behavior following peripheral nerve injury and inflammation, and this extensive body of work is covered comprehensively by several recent reviews (Ellis and Bennett, 2013; Ji et al., 2013; Mika et al., 2013). Importantly, spinal injection of astrocytes that had been activated by TNFα was shown to be sufficient to produce mechanical hypersensitivity in uninjured animals (Gao et al., 2010). Far fewer studies have been made of astroglial contributions to neuropathic pain caused by SCI, but the findings thus far show interesting similarities to what has been described in peripheral neuropathic pain models. Like microglia, astrocytes proliferate after SCI, especially in the region of the lesion, forming a glial scar (Byrnes and Faden, 2007; Karimi-Abdolrezaee and Billakanti, 2012; Zai and

Wrathall, 2005). This proliferation and the activation of astroglia (reactive gliosis) by SCI (e.g., O'Brien et al., 1994; Popovich et al., 1997; Baldwin et al., 1998; Carlton et al., 2009; Gwak and Hulsebosch, 2009) may be even more pronounced than the various forms of activation reported for microglia after SCI, as has also been noted in models of peripheral injury and inflammation (Ji et al., 2013). Reactive gliosis is typically assessed by upregulation of GFAP, which is a rapid and relatively specific indicator of astrocyte activation. Using both immunostaining and Western blot to measure GFAP expression, an early and particularly informative study showed that thoracic spinal contusion caused astrocyte activation not only at the lesion site but throughout the spinal cord, and striking increases in GFAP remained for as long as the investigators assayed the cord (up to 9 months after SCI) (Nesic et al., 2005). Importantly, this same study showed that the changes in GFAP expression were correlated with mechanical hypersensitivity of hindpaws and forepaws, and showed that other proteins preferentially expressed in astrocytes or likely to be involved in pain-related functions of reactive astrocytes were also upregulated a month or later after SCI. These included the astrocytic Ca^{2+}-binding protein S-100 and the water channel protein aquaporin 4 (implicated in inflammatory edema in the CNS, which probably involves the extensive contacts made by astrocytes onto spinal blood vessels, Fukuda and Badaut, 2012).

Efforts to define contributions of astrocytic mechanisms to SCI pain have been limited by a lack of specific inhibitors of astrocyte activity. Preclinical studies of SCI pain have described suppression of reflex hypersensitivity by drugs that target astrocytes but also microglia and other cell types, notably propentofylline (Gwak and Hulsebosch, 2009; Gwak et al., 2008; Gwak et al., 2009) and also ibudilast (Hama et al., 2012). In peripheral nerve injury models pain has been associated with relatively high activity of c-Jun N-terminal kinase (JNK) in astrocytes compared to neurons or microglia (Zhuang et al., 2006). Recently it was reported that contusive SCI at T10 caused activation of JNK in lumbar segments lasting at least a month, and that mechanical and heat hypersensitivity of hindlimb reflexes was reduced by intrathecal injection of the JNK inhibitor, SP600125 (Lee et al., 2013). Interestingly, the same study found similar suppressive effects produced by acupuncture. Perhaps the most compelling evidence for essential roles of astrocytes in neuropathic SCI pain has come from investigations of another feature of astrocytes that is not shared with microglia: coupling by gap junctions, which depends upon connexin 43 (Cx43). Transgenic mice with deletion of Cx43 showed reduced GFAP expression 1-2 months after contusive SCI and little or no mechanical or heat hypersensitivity of paw withdrawal (Chen et al., 2012). Interestingly, this study showed that suppression of the persistent hypersensitivity was much more dramatic after Cx43 knockout than after early

minocycline treatment (similar to that used in rats by Marchand et al., 2005 and Tan et al., 2009), suggesting a larger role for reactive astrocytes than microglia in the development of neuropathic SCI pain, at least in mice. Consistent with a major role for intracellular coupling among astrocytes in the initial development of SCI pain, early but not late treatment of rats with the gap junction decoupler, carbenoxolone, reduced later mechanical and heat hypersensitivity, as well as GFAP staining after spinal hemisection (Roh et al., 2010).

Reactive gliosis after SCI persists for as long as has been examined in animal models (Gwak et al., 2012; Wu et al., 2012). Inhibition of proliferation in general (and possibly of other cellular effects in non-proliferating neurons) by a cyclin-dependent cyclase (CDK) inhibitor, CR8, was found to produce parallel reductions in lesion volume, in SCI-induced upregulation of cell cycle-related proteins in astrocytes and microglia, in the elevated expression of GFAP and Iba-1, and in various signs of central inflammation months after SCI (Wu et al., 2012). Interestingly, these effects and concomitant neuropathic SCI pain appear to depend upon a neurotrophin, BDNF, that is associated with many forms of neural plasticity, including pain-related plasticity in peripheral injury models (Merighi et al., 2008), and which prominently involves ATP-induced release of BDNF from microglia (Coull et al., 2005; Trang et al., 2011). Neuronal effects of microglial BDNF release mediated by trkB receptors have been emphasized in peripheral neuropathic pain models. However, BDNF effects are also mediated through an alternatively spliced truncated form of the BDNF receptor, trkB.T1, which is expressed in astrocytes, oligodendrocytes, and Schwann cells as well as in neurons (Fenner, 2012) and has been shown to be important for neuropathic SCI pain. An extensive study (Wu et al., 2013b) found upregulation of trkB.T1 for at least 2 months at a thoracic contusion site (see also Liebl et al., 2001) and at least 3 days in the distant lumbar enlargement in wild-type mice. Mice with genetic deletion of trkB.T1 showed significant reduction of mechanical hypersensitivity in hindlimb tests, which was associated with enhanced recovery of motor function, a smaller lesion volume, less GFAP and Iba-1 expression, persistent downregulation of genes and proteins in cell cycle pathways, and less elevation of cell cycle expression in reactive astrocytes assayed with an *in vitro* model system. Deletion of trkB.T1 occluded the suppressive effects of the CDK inhibitor on mechanical hypersensitivity and motor recovery after SCI (Wu et al., 2013b). These intriguing findings point to important roles for BDNF, trkB.T1, and cell cycle-related proteins in neuropathic SCI pain, but much remains to be learned about where and how these molecules contribute to neuroinflammation, glial proliferation, and pain after SCI.

In sum, observations from SCI pain models, supported by a larger body of evidence from peripheral neuropathic pain models (e.g., Zhuang et al., 2005; Colburn et al., 1999; Sweitzer et

al., 1999) indicate that interactions among astrocytes, microglia, and neurons are critical for the development and maintenance of neuropathic SCI pain (Gwak et al., 2012; Ji et al., 2013). Important interactions probably also involve satellite glial cells in sensory ganglia, which are closely related to astrocytes and are known to contribute to behavioral hypersensitivity in peripheral models of neuropathic pain (Huang et al., 2013; Ji et al., 2013; Ohara et al., 2009; Xie et al., 2009), but contributions of satellite glial cells to painful consequences of SCI have yet to be reported.

Is neuropathic SCI pain driven by a unified host defense system?

An implicit assumption guiding most work on neuropathic SCI pain is that the pain arises in a disorganized fashion from any of numerous, often independent effects of the injury on different components of pain pathways (see section above on classes of mechanisms). An interesting possibility is that SCI also inadvertently activates integrated response systems that employ pain and neuroinflammation as part of host defense. It has long been appreciated that many interactions occur between the innate immune system and the peripheral nervous system, with peripheral neurogenic inflammation being especially prominent (Richardson and Vasko, 2002). Compelling arguments have been made for viewing the immune and somatosensory nervous systems as composing a unified system that functions in host defense, broadly conceived as integrating perceptual and behavioral responses (pain behavior) with classical responses of the innate and adaptive immune systems (Chiu et al., 2012). By this view, primary nociceptors (which strongly activate pain pathways) are the first responders for host defense, initiating not only rapid perceptual and behavioral responses but also early inflammatory responses (neurogenic inflammation) to tissue injury and infection. This broad sentinel role accounts for the expression in C-fiber nociceptors of numerous pattern recognition receptors (PRRs) for damage-associated molecular patterns (DAMPs or alarmins, intracellular molecules such as high-mobility-group box 1 [HMGB1] and heat shock proteins released by ruptured or necrotic cells) and pathogen-associated molecular patterns (PAMPs, e.g., components of bacterial and yeast cell wall components and viral RNA), as well as receptors for intense mechanical stimulation and for signs of ongoing inflammation (Fig.1). In C-fiber nociceptors under normal, inflamed, and/or injured conditions these often include TRP channels (especially TRPV1 and TRPA1), cytokine and prostaglandin receptors (e.g., IL-1β–R, TNFα-R, IL-6-R, EP4), chemokine receptors (e.g., CCR2 for MCP-1/CCL2), PRRs (e.g., toll-like receptors [TLRs] 3, 4, 7, and 9, Nod-like receptors [NLRs] and receptor for advanced glycation endproducts [RAGE]), receptors

for other intracellular constituents released during injury (P2X3 and P2Y ATP receptors, AMPA and NMDA glutamate receptors), and receptors for growth factors (e.g., trkA for nerve growth factor [NGF] and trkB for BDNF) (Chiu et al., 2012; Miller et al., 2009; Shibasaki et al., 2010). In addition, primary nociceptors can be sensitized and activated by other effector molecules in the innate immune system important in inflammation, including the complement fragments C5a and C3a, suggesting the presence of complement receptors on these neurons (Jang et al., 2010). Most, if not all, of these diverse receptors are expressed on peripheral and central terminals, where they modulate the gain of nociceptor inputs and outputs, respectively, and also on nociceptor somata in the DRG, where they may detect blood-borne danger signals unimpeded by a significant vascular permeability barrier (Abram et al., 2006; Jimenez-Andrade et al., 2008). Primary nociceptors communicate directly with cells in the innate immune system (including peripheral macrophages, neutrophils, and T cells, and central microglia and astrocytes) by releasing glutamate, ATP, neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP), chemokines such as CCL2, CCL21, and CX3CL1 (fractalkine), cytokines such as IL1β, IL-6, and TNFα, and growth factors such as BDNF, neuregulin 1 (NRG1), and basic fibroblast growth factor (bFGF) (Calvo and Bennett, 2012; Chiu et al., 2012; Ji et al., 2013; McMahon and Malcangio, 2009; Miller et al., 2009; Pezet and McMahon, 2006) (Fig.1). An integral role of primary nociceptors in innate immunity recently received unexpected affirmation by the discovery that bacteria can directly activate these neurons without mediation by known immune cells (Chiu et al., 2013).

This broader biological view raises the interesting possibility that SCI generates a complex set of signals that are detected by primary nociceptors, which if then sufficiently activated, drive pain as part of a unified host defense response. A complementary view is that persistent central and peripheral signals generated by SCI mimic the pattern that would be generated by very severe peripheral injury, and this induces a long-lasting hyperfunctional state in numerous primary nociceptors that would normally serve to compensate for loss of peripheral sensory function and to protect body regions made more vulnerable by debilitating injury (Walters, 2012). In particular, nociceptors may be sensitive to central as well as peripheral inflammatory signals (integrating these with other signals of severe bodily injury, such as retrograde signals from intensely activated postsynaptic neurons, Walters, 2012), and nociceptor activity may in turn stimulate central as well as peripheral inflammatory responses (see below). An implication of these views is that positive feedback loops between enhanced electrical activity in primary nociceptors and activation both of peripheral and of central inflammatory cells may help to

sustain neuroinflammation and chronic neuropathic pain (Miller et al., 2009; Walters, 2012; Xie et al., 2009).

Support for these views first came from evidence that contusive SCI enhances the growth of uninjured nociceptors distant from a spinal lesion site (Bedi et al., 2012; Hou et al., 2009; Krenz and Weaver, 1998; Ondarza et al., 2003; Ramer et al., 2012). SCI was then found to enhance peripheral function in C-fiber nociceptors, with sensitivity to mechanical and heat stimuli being increased in a forepaw skin-nerve preparation 5 weeks after T10 contusion (Carlton et al., 2009). Significantly, this study found that spontaneous electrical activity (SA) was generated at a low rate in the peripheral terminals of nociceptors after SCI. Nociceptor SA induced by SCI was also found to be generated in the soma in the dorsal root ganglion (DRG) in vivo and in vitro (Bedi et al., 2010). Intrinsic SA and hyperexcitability were present in ~50% of small neurons dissociated from DRGs below and at (but not above) the T10 injury level, and the high incidence remained unchanged for at least 5 months after SCI. Importantly, the intrinsic SA was correlated with mechanical and heat hypersensitivity of hindlimb and forelimb withdrawal responses, as well as with increased incidence of a supraspinally mediated response, vocalization, evoked at but not below the injury level (Bedi et al., 2010) -- a pattern like that reported by many SCI patients (Finnerup, 2012).

Most of the dissociated DRG neurons showing SA after SCI were responsive to the specific activator of TRPV1, capsaicin (Bedi et al., 2010), and TRPV1 expression was increased in lumbar DRGs 4-6 weeks after thoracic contusion (Wu et al., 2013c) (see also DomBourian et al., 2006; Ramer et al., 2012; Zhou et al., 2002). Very low concentrations of capsaicin (10 nM) produced non-desensitizing, non-accommodating repetitive firing in dissociated nociceptors indistinguishable from SCI-induced SA, and this effect and other cellular responses to capsaicin were enhanced by prior SCI (Wu et al., 2013c). Most important, SCI-induced mechanical and heat hypersensitivity of hindlimb withdrawal responses was reversed by antisense knockdown of TRPV1 or by systemic injection of a specific TRPV1 antagonist, AMG9810 (Wu et al., 2013c) (see also Rajpal et al., 2007). While TRPV1 channels have been observed in other cells, they are expressed most abundantly in nociceptors (Caterina et al., 2000; Lauria et al., 2006), supporting the possibility that interruption of nociceptor SA contributed to these suppressive effects. A major role for nociceptor activity after SCI was also indicated by reversal of SCI-induced reflex hypersensitivity by knockdown of a voltage-gated Na^+ channel, Nav1.8 (Yang et al., 2012) that is primarily expressed in primary somatosensory neurons, including >90% of C-fiber nociceptors (Liu and Wood, 2011; Shields et al., 2012). TRPV1 has important functions in host defense, being activated and/or sensitized by many features of inflammation, including

acidity, numerous lipids generated during cellular injury or ischemia, and a growing number of other injury-related molecules (amines, ATP, NO, reactive oxygen species [ROS], CCL2) (Jung et al., 2008; Miyamoto et al., 2009; Morales-Lazaro et al., 2013; Nishio et al., 2013). Thus, after SCI, TRPV1 receptors may detect multiple signs of neuroinflammation both peripherally and in the spinal cord – specifically, in the central processes of nociceptors and/or in TRPV1-expressing spinal neurons (Kim et al., 2012).

The somata of primary nociceptors may be an important locus for detecting inflammatory signals and integrating them with other signals of serious bodily injury (Walters, 2012). Pain-promoting sensitization of DRG neurons is known to occur after experimental inflammation around a ganglion (Wang et al., 2007; Xie et al., 2006), which causes upregulation of TRPV1 in nociceptors and the generation of numerous cytokines in the DRG (Dong et al., 2012; Strong et al., 2012). After SCI, nociceptor somata are exposed to macrophages and T cells that infiltrate into DRGs close to and distant from a spinal lesion (McKay and McLachlan, 2004). The relatively ineffective vascular permeability barrier of DRGs (Abram et al., 2006; Jimenez-Andrade et al., 2008) and normal exposure of DRG neurons to cerebrospinal fluid (CSF) means that nociceptor somata will be highly exposed to the elevated levels of cytokines that have been observed in the blood (Davies et al., 2007; Stein et al., 2013) and CSF (Kwon et al., 2010) of SCI patients. One of these cytokines, macrophage migration inhibitory factor, MIF, is secreted by leukocytes and the anterior pituitary gland, and is constitutively expressed in many other cells, including microglia and DRG neurons (Alexander et al., 2012; Bucala, 1996; Wang et al., 2010). MIF is particularly interesting because the circulating concentration of MIF is normally ~1000 fold higher than other pro-inflammatory cytokines (Aloisi et al., 2005; Bucala, 1996; Calandra and Roger, 2003), and this concentration was doubled in SCI patients compared to uninjured controls (Stein et al., 2013). Importantly, the concentration of MIF in SCI patients, ~1 ng/ml, was the same as shown to increase the excitability of a subset of putative nociceptors isolated from mouse DRGs (Alexander et al., 2012). This latter study also showed that MIF-null mice fail to develop pain after nerve injury or hindpaw inflammation, suggesting that MIF function is essential for both neuropathic and inflammatory types of pain, extending previous findings in rats (Wang et al., 2010; Wang et al., 2011). Furthermore, MIF application increased expression of TNF-α, IL-1β, IL-6, CCL2, and iNOS in mouse and rat microglia, and MIF increased neurite outgrowth in isolated mouse DRG neurons (Alexander et al., 2012). All of these effects have also been observed after SCI (see above). The actions of MIF on microglia suggest that it could contribute to neuroinflammation and pain after SCI by central actions on microglia as well as by peripheral activation of nociceptors, raising the intriguing possibility that

MIF has a key role in integrating painful neuroinflammatory responses of a unified host defense system to SCI. SCI also releases stress hormones (Fig. 1), such as glucocorticoids into the circulation, and these can result in immunosuppression, potentially opposing neuroinflammatory responses to SCI (Lucin et al., 2009). Interestingly, glucocorticoids induce MIF (Flaster et al., 2007), suggesting that coordinated upregulation of MIF may function to preserve or enhance pain sensitivity during stressful conditions, such as SCI, when glucocorticoids suppress many other aspects of immune function (Alexander and Popovich, 2009).

Inflammatory responses to peripheral injury that occur both in DRGs and in the spinal cord depend upon electrical activity in primary afferent neurons (Hathway et al., 2009; Thacker et al., 2009; Van Steenwinckel et al., 2011; Wen et al., 2007; Xie et al., 2009). An important implication of this observation should be emphasized. If primary nociceptors function as part of the host defense system, then the central neuroinflammatory responses evoked by nociceptor activity may also represent a host defense function, at least under some conditions. Thus, from a broader biological perspective, central neuroinflammation may not always be maladaptive; limited nociceptor-evoked spinal neuroinflammation might, for example, be a mechanism that helps maintain adaptive pain targeted to a severely injured body part (Walters, 2012). Central neuroinflammation driven by nociceptor activity after SCI may be especially important in regions distant from a spinal injury site, where there would be much less damaged tissue generating DAMPs and other injury signals to drive local inflammation. Although it has been suggested that C-fiber nociceptors may be less important than other primary afferents for driving central neuroinflammation after peripheral nerve injury (Suter et al., 2009), this inference was based on sciatic nerve block methods that would not have reduced persistent SA generated in nociceptor somata in the DRG proximal to the block. Taken together, the studies reviewed in this section support the hypothesis that primary sensory neurons, including C-fiber nociceptors, are an integral part of a unified host defense system that can drive both peripheral (Chiu et al., 2012) and central inflammatory responses, and they support the possibility that this system may be activated after SCI to help drive neuropathic SCI pain (Fig. 1). Of course, the host defense system evolved to produce adaptive pain after peripheral injury and inflammation, so it should also be important for driving the second general class of pain endured by SCI patients – nociceptive pain triggered by overuse and by other secondary consequences of SCI for peripheral tissues.

Implications of neuroinflammatory mechanisms for treating neuropathic SCI pain

No front-line treatments currently used for neuropathic SCI pain specifically target neuroinflammatory mechanisms, although they may do so indirectly. Standard treatments for SCI pain are based on those commonly (and with only limited success) used for peripheral neuropathic pain, notably the anticonvulsants gabapentin and pregabalin, and antidepressants such as amitriptyline, although many other drugs are used, including other serotonin-norepinephrine reuptake inhibitors, opioids, and intrathecal delivery of clonidine and ziconotide (Finnerup and Baastrup, 2012). Only pregabablin has been approved by the FDA for the treatment of neuropathic SCI pain, while other drugs used for this purpose were approved for other uses. Pregabalin has shown partial efficacy in two large-scale, randomized, placebo-controlled clinical trials (Cardenas et al., 2013; Siddall et al., 2006). Smaller randomized controlled trials have also indicated partial efficacy for gabapentin (Teasell et al., 2010), which shares mechanisms of action with pregabalin. A randomized controlled trial has shown significant but partial efficacy of amitriptyline in depressed but not non-depressed SCI patients (Rintala et al., 2007). Other commonly used drugs either have exhibited very little or no efficacy in clinical trials (e.g., lamotrigine), present major problems for long-term use (e.g., i.v. ketamine, i.v. lidocaine), or have not yet been tested rigorously in clinical trials for SCI pain (oral opioids, oral ketamine) (Teasell et al., 2010). Importantly, no drugs have demonstrated high efficacy against neuropathic SCI pain, and all have significant adverse side effects (Finnerup and Baastrup, 2012; Teasell et al., 2010).

Given the findings from animal models reviewed above, therapeutic approaches explicitly targeting neuroinflammatory mechanisms would be logical alternatives or complements to existing treatments for neuropathic SCI pain. Early evidence suggests that finding such treatments may be possible but challenging, as indicated by disappointing results in clinical trials for some of the agents that seemed quite promising in preclinical models of neuropathic pain. Treatment of patients with IL-10 illustrates general problems that can prevent the therapeutic use of an agent that effectively reduces neuroinflammation in both animal models and humans. On the basis of its powerful anti-inflammatory effects in a variety of animal models (including models of rheumatoid arthritis, diabetes, and inflammatory bowel disease), administration of IL-10 by direct injection, viral delivery, or adoptive transfer of IL-10-secreting cells appeared to offer exciting potential for treating many clinical conditions, including neuropathic pain (Milligan et al., 2012). However, repeated injections of IL-10 in various clinical trials has failed to improve disease symptoms and has revealed serious adverse effects, including a marked reduction in red blood cell counts (Bijjiga and Martino, 2013). Furthermore,

trials with gene therapy strategies that could produce more sustained elevations of IL-10 levels have not been attempted because of concern about potential dangers of prolonged immune suppression, including chronic infections and increased likelihood of certain cancers, as well as changes in cytokine balance that can increase allergic responses and asthma (Bijjiga and Martino, 2013). Moreover, a significant potential problem after SCI is that general suppression of inflammation may impair regeneration and repair in the spinal cord (Benowitz and Popovich, 2011). Nevertheless, the strategy of harnessing endogenous anti-inflammatory signals to combat neuropathic SCI pain is appealing. Anti-inflammatory, pro-resolution lipid signals such as resolvins, protectins/neuroprotectins, and lipoxins that have shown efficacy in preclinical models of peripheral neuropathic pain (Ji et al., 2011; Serhan et al., 2008) should offer promising candidates to investigate in the context of neuropathic SCI pain.

Other agents that reduce neuroinflammation and pain in rodent SCI models have either failed to alleviate neuropathic pain in clinical trials or have not yet been tested for effects on neuropathic pain. The nonspecific glial inhibitor, propentfylline, failed to decrease pain in post-herpetic neuralgia patients (Lau et al., 2012). An unsettling note for preclinical studies of neuroinflammation is that this clinical failure may have reflected unexpected differences between human and rodent microglial properties, with human microglia being less responsive to a potent inflammogen, lipopolysaccharide (LPS), and to propentofylline than are rat microglia (Lau et al., 2012). Another nonspecific glial inhibitor, ibudilast, has demonstrated safety but only weak evidence of neuroprotection in a multiple sclerosis trial (Barkhof et al., 2010), and no results on efficacy against pain have been reported. In a small preliminary trial, the nonspecific microglial inhibitor, minocycline, failed to reduce pain caused by capsaicin application in patients with unilteral sciatica, but a trend was noted to improve ongoing pain prior to the capsaicin test (Sumracki et al., 2012). Larger clinical trials are underway to investigate minocycline's efficacy in treating pain associated with peripheral nerve damage (ClinicalTrials.gov: NCT01869907) and intercostal neuralgia (ClinicalTrials.gov: NCT01214482). Weak clinical results were also reported for a blocker of the chemokine, CCL2; an antagonist, AZD2423, of the CCR2 receptor showed no efficacy on primary pain variables after post-traumatic neuralgia, although somewhat encouraging trends were noted in subscores for paroxysmal pain and paresthesia/dysesthesia (Kalliomaki et al., 2013). Other plausible approaches to reduce neuroinflammation-associated pain have not yet been tested in clinical trials on neuropathic pain. Licofelone, which inhibits both COX and LOX enzymes in the arachidonic acid cascade, has shown effectiveness in clinical trials on arthritis, albeit with mixed results on pain (Raynauld et al., 2009; Wildi et al., 2010). Arthritic pain and central neuroinflammatory pain may differ in critical underlying

mechanisms, so the possibility remains that neuropathic SCI pain will be sensitive to licofelone. A second potentially beneficial effect of licofelone was observed in a rodent SCI model: it reduced p-glycoprotein-mediated drug resistance (Dulin et al., 2013b), suggesting that dual inhibitors of COX and LOX, such as licofelone, might simultaneously reduce neuroinflammation, ameliorate neuropathic pain, and improve bioavailability of other therapeutic drugs in SCI patients. Another logical target for therapeutic drug development is MIF, the inhibition of which may have potent effects on neuropathic pain (Alexander and Popovich, 2009), although its role in neuropathic SCI pain has not yet been demonstrated.

A novel but untested approach to treating neuropathic SCI pain may be to target ongoing activity in primary nociceptors. This strategy is suggested by indications that primary nociceptors are an integral part of a host defense system that can contribute to central as well as peripheral inflammation (and consequent pain), and by preclinical evidence that persistent activity in nociceptors drives behavioral hypersensitivity after SCI (Wu et al., 2013c; Yang et al., 2012). These preclinical studies found that interventions that reduced TRPV1 or Nav1.8 function sufficiently to eliminate spontaneous activity in nociceptors, but not to block reflex responses evoked by mechanical or heat stimuli, were effective in reversing behavioral hypersensitivity. This suggests that central sensitization and neuropathic SCI pain might be attenuated selectively by using more prolonged treatment with lower doses of TRPV1 antagonists (which, unlike Nav1.8 antagonists, have been tested successfully in humans) than tried thus far in clinical trials (Chizh et al., 2007; Krarup et al., 2011; Krarup et al., 2013; Rowbotham et al., 2011; Schaffler et al., 2013). A new generation of potent and selective TRPV1 antagonists is becoming available (e.g., Reilly et al., 2012) that lack the hyperthermic side effects of previous antagonists (Gavva et al., 2008). If interventions that block ongoing activity of nociceptors and reduce reflex hypersensitivity are found also to block operant indications of chronic, ongoing pain in rodent models, this would provide a strong impetus for testing whether similar drugs reduce ongoing pain in SCI patients.

Conclusions

While multiple mechanisms contribute to neuropathic pain after SCI, numerous experimental observations indicate that persistent neuroinflammation is critical for the development and maintenance of this pain. Most of these observations are recent; only within the last decade has it become clear that neuroinflammation after SCI is chronic (perhaps permanent) and that interventions that target persistent neuroinflammation ameliorate behavioral hypersensitivity in

animal models of neuropathic SCI pain. A limitation in nearly all of the animal studies is a reliance on spinally-mediated reflexive measures of pain, usually enhancement of hindlimb withdrawal responses, which bear an uncertain relationship to the cortically-mediated states having emotional and cognitive components that are central to the human pain experience. Nevertheless, neuroinflammation and behavioral hypersensitivity after SCI have been linked to altered activity in several components of classical pain pathways that would be expected to promote pain, including primary afferent neurons, dorsal horn neurons, and thalamic neurons. Studies have shown correlations between SCI-induced reflex hypersensitivity and several measures of activation and proliferation by microglia (plus infiltrating macrophages) and astrocytes, along with parallel reversals of behavioral and glial alterations by interventions designed to reduce neuroinflammation. The interventions that have appeared successful in reducing SCI pain include treatments with agents that inhibit the activation of microglia and/or astroglia (IL-10, minocycline, etanercept, propentofylline, ibudilast, licofelone, SP600125, carbenoxolone), pharmacological (CR8) and/or genetic disruption of cell cycle-related proteins or a truncated receptor (trkB.T1) for BDNF, and reduction in the activity of channels (TRPV1 and Nav1.8) important for electrical activity in primary nociceptors by antisense knockdown or pharmacological inhibition (AMG9810). Evidence that chronic activity in primary nociceptors contributes to neuropathic SCI pain, evidence that nociceptor activity drives central neuroinflammation in peripheral injury models, and increasing support for the idea that nociceptors function within a unified host defense system all suggest that spinal and systemic effects of SCI can activate nociceptor-mediated host defense responses that interact with complex central consequences of SCI to drive chronic pain. This broader view of SCI-induced neuroinflammation may aid in the identification of new targets for treating SCI pain.

Acknowledgements

ETW acknowledges support from the Department of Defense USAMRAA, Craig Neilsen Foundation, Mission Connect/TIRR, and National Science Foundation during the preparation of this article.

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Figure Legend

Fig. 1. Extracellular signaling involved in SCI-induced neuroinflammation and neuropathic pain, including a nociceptor-mediated link between peripheral host defense and central inflammation. The highly schematic diagram depicts some of the complex interactions among glial cells, infiltrating leukocytes (only macrophages and T cells are indicated, but other types are also involved), and neurons that have been implicated in the development and maintenance of neuropathic SCI pain. Spinal cord and dorsal root ganglia (DRG) are shown, but some of the interactions important for pain occur in supraspinal components of the pain pathway as well (see text). Listed below the schematic diagram are some of the inflammatory signals known to stimulate (inputs) or be released by (outputs) each of three cell types prominently involved in neuroinflammation and neuropathic pain (on the basis of evidence from both central and peripheral injury models): astrocytes, microglia, and nociceptors. Note that each list is incomplete and that some of the listed signals are not constitutively present, instead being conditionally induced in a given cell type (often by inflammation or injury). Signal abbreviations are defined in the text.

Figure

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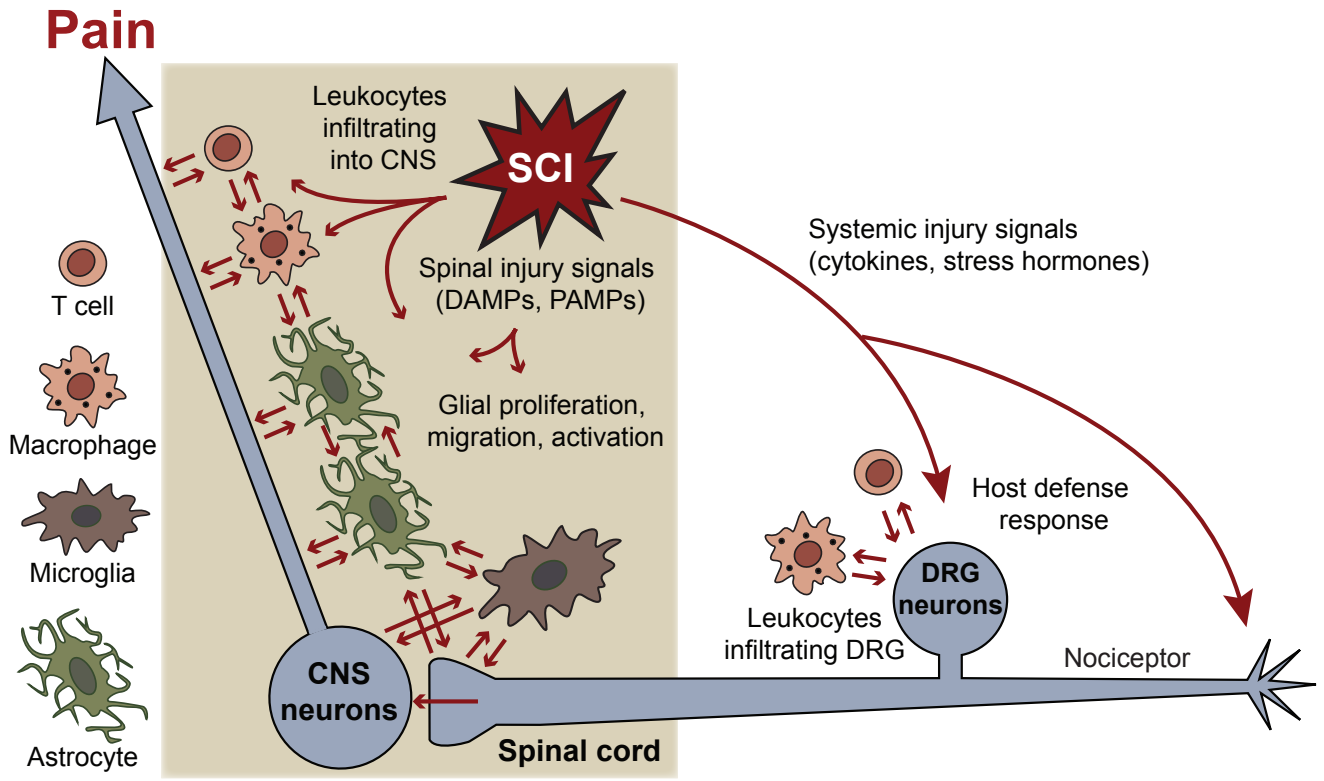

Inflammatory signals that may contribute to neuropathic SCI pain

Astrocytes

Inputs	Outputs
ATP	ATP
DAMPs, PAMPs (TLRs)	Glu
Glu	IL1-β
TNF-α	IL-6
IL-18	CCL2
SP	IL-10
BDNF (trkB.T1)	bFGF
bFGF	D-serine
IL-10	NO
	MMP-2

Microglia

Inputs	Outputs
ATP	ATP
DAMPs, PAMPs (RAGE, TLRs)	BDNF
IL1-β	TNF-α
IL-18	IL1-β
CCL2	IL-6
CCL21	IL-18
IFN-γ	CCL2
MIF	IL-10
SP	PGE_2
CGRP	NO
bFGF	Cathepsin S
NRG1	
IL-10	

Nociceptors

Inputs		Outputs
ATP	TNF-α	ATP
DAMPs, PAMPs (RAGE, TLRs)	IL1-β	Glu
	IL-6	CGRP
Complement (C5a, C3a)	CCL2	SP
	IL-18	TNF-α
Glu	MIF	IL1-β
PGE_2	BDNF	IL-6
Lipid signals (TRPV1)	bFGF	CCL2
		CX3CL1
H^+ (TRPV1, ASIC)		MIF
ROS, NO (TRPV1, TRPA1)		bFGF
		NRG1
		NO
		MMP-9

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